Analysis of the Pathogenesis of Severe Anaemia of Pigs due to *Mycoplasma suis* Infection

---

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Kathrin M. Felder

von

Entlebuch LU

Promotionskomitee

Prof. Dr. Leo Eberl (Vorsitz)

PD Dr. Ludwig E. Hölzle (Leitung der Dissertation)

Prof. Dr. Jakob Pernthaler

Prof. Dr. Max M. Wittenbrink

Zürich, 2010

Table of Contents

1. Summary

Infectious anaemia of pigs (IAP) is a disease caused by the haemotrophic bacterium *Mycoplasma suis*. The infection goes along with acute haemolytic anaemia, severe hypoglycaemia, bacteraemia and elevated body temperature. Antibiotic treatment by means of oxytetracycline saves animals from death but mycoplasmas are not eradicated. The disease goes over to the chronic state characterised by milder anaemia, acrocyanosis, higher susceptibility to other infections and growth retardation of piglets leading to economic consequences for affected farmers. Haemotrophic mycoplasmas are not cultivable *in vitro*. Therefore, infections with *M. suis* often are not recognised. These latently infected animals provide a reservoir and are of epidemiologic significance. *M. suis* is passed on to animals by transmission of infected blood mainly due to mechanical manipulations or by blood sucking arthropods.

A main clinical sign of IAP is the autoimmune haemolytic anaemia. Due to drawbacks in research, i.e. the unculturability *in vitro*, not much is known about its pathogenesis. Clarification of mechanisms that lead to illness would support the establishment of treatment procedures and of a protective vaccination. Earlier studies revealed the presence of cold reactive antibodies targeting red blood cells. These mainly occur during the chronic phase and are responsible for the cyanosis observed at the acra. Agglutination of the red blood cells in the periphery leads to cell lysis and decreased haematocrit levels. In addition, autoreactive antibodies of the isotype IgG are upregulated during phases with acute clinical signs. In this thesis, actin was identified as target protein of these warm reactive antibodies.

The normal life span of a red blood cell is about 120 days. To prevent haemolysis and inflammation senescent red blood cells are safely removed from the circulation system by means of programmed cell death (eryptosis). Breakdown of membrane asymmetry represents a signal for macrophages to ingest the affected cell and to transport it to the spleen or liver for sequestration. Programmed cell death is as well induced upon stress. An example is malaria. Cells infected with *Plasmodium falciparum* are recognised and eliminated by macrophages. Here, eryptosis

represents a defence mechanism against intracellular pathogens. In this thesis, the occurrence of eryptosis in *M. suis* infected pigs was described and evaluated for impact on anaemia development. The pattern of eryptosis occurrence depends on the *M. suis* strain. In infections with strains of low virulence, eryptosis is observed at the beginning of infection and seems to protect the animal from severe anaemia by removal of infected cells from the circulation system. In fact, in pigs infected with a highly virulent strain eryptosis and severity of anaemia correlate. Due to high numbers of cleared cells severe anaemia occurs. Later on, intravascular lysis due to another mechanism was observed.

The anaemia is most distinct at time points of maximal bacteraemia indicating that the direct interaction of *M. suis* with its host cell is important for development of anaemia. Attachment is a prerequisite for *M. suis* to invade the red blood cell. By doing so, significant deformations and destructions are induced leading to cell lysis. A promising approach to ease the clinical sign of anaemia would be to prevent this attachment process. For this, knowledge of attachment proteins is indispensable. MSG1, a protein homologous to glyceraldehyde-3-phosphate dehydrogenase was shown earlier to be involved in the attachment process. The protein was used to vaccinate pigs prior to experimental infection with *M. suis*. However, no protection was achieved.

In vitro studies performed in connection with this doctoral thesis indicated that actin was the interaction partner of MSG1 on the red blood cell. Interaction with actin would lead to rearrangement and conformational changes of the protein which could induce the aforementioned misled immune response directed against host actin.

2. Zusammenfassung

Die infektiöse Anämie des Schweins (IAP) wird durch *Mycoplasma suis* verursacht. Symptome sind akute immun-hämolytische Anämie, starke Hypoglykämie, Bakteriämie und hohes Fieber. Eine Behandlung mit Oxytetrazyklin verhindert, dass die Schweine sterben, vermag aber nicht, den Erreger komplett aus dem Organismus zu eliminieren. Die Krankheit geht in ein chronisches Stadium über, das durch leichtere Anämie, Zyanosen an den Akren, ein erhöhtes Risiko für andere Infektionskrankheiten und Wachstumsverzögerungen der Ferkel gekennzeichnet ist. Die Symptome während der chronischen Phase sind hauptsächlich für die wirtschaftlichen Schäden verantwortlich. *M. suis* gehört zu der Gruppe der haemotrophen Mykoplasmen, die im Labor nicht kultivierbar sind. Daher werden sie in der Routinediagnostik oft nicht erkannt. Gesunde Tiere werden mit *M. suis* infiziertem Blut angesteckt was vor allem iatrogen oder durch blutsaugende Insekten erfolgen kann.

Ein Hauptsymptom der IAP ist die autoimmunhämolytische Anämie, über deren Pathogenese fast nichts bekannt ist. Der Grund dafür liegt an der Unkultivierbarkeit von *M. suis*, was die Forschung deutlich erschwert. Eine Aufklärung der Pathogenitätsmechanismen wäre sehr hilfreich für die Verbesserung von Therapiemöglichkeiten und die Entwicklung eines Impfstoffs. In früheren Studien wurde gezeigt, dass während der chronischen Phase Kälteagglutinine gebildet werden. Hierbei handelt es sich um autoreaktive Antikörper, die bei erniedrigter Körpertemperatur an die Erythrozyten binden und diese so agglutinieren. Dies führt zu einer Behinderung des Blutflusses in den Kapillaren und daher zu Zyanosen an den Extremitäten, insbesondere an den Ohrrändern. Eine Bindung der Kälteagglutinine an die Erythrozyten führt zu Agglutination und Zelllyse gefolgt von einer Erniedrigung des Hämatokrits. Zusätzlich zu den Kälteagglutininen werden während der akuten Krankheitsstadien autoreaktive IgG Antikörper gebildet. In dieser Dissertation wurde Aktin als Autoantigen für die IgG Antikörper identifiziert.

Die normale Lebensdauer eines Erythrozyten beträgt ungefähr 120 Tage. Nach dieser Zeit wird die Zelle durch den Mechanismus des programmierten Zelltods aus dem Blutkreislauf entfernt ohne Entzündungsreaktionen oder Hämolyse auszulösen.

Dieser Prozess wird in Analogie zur Apoptose anderer Zellen Eryptose genannt. Durch äussere Signale wird die Membranasymmetrie aufgehoben, was zur Aufnahme durch Makrophagen führt. Diese transportieren die Zelle zur Milz oder Leber, wo sie dann abgebaut wird. Bei Malaria dient die Eryptose als Verteidigungsmechanismus, da Zellen, die mit Plasmodien infiziert sind, gezielt abgebaut werden. In der vorliegenden Doktorarbeit wurde das Auftreten der Eryptose in mit *M. suis* infizierten Schweinen untersucht und in den Zusammenhang mit der Anämiepathogenese gestellt. Bei Tieren, die mit einem wenig virulenten *M. suis* Stamm infiziert wurden, konnten Eryptoseprozesse zu Beginn der Infektion erkannt werden. Die Tiere zeigten keine Anämie und überlebten ohne Behandlung, was darauf hindeutet, dass *M. suis* infizierte Blutzellen sofort aus dem Blutkreislauf entfernt wurden bevor Symptome entstehen konnten. Bei Schweinen, die mit einem virulenteren *M. suis* Stamm infiziert waren, konnte eine Hochregulierung der Eryptose-Aktivität beobachtet werden, die mit dem Schweregrad der Anämie korrelierte. Da sehr viele Zellen mit *M. suis* infiziert sind und daher mittels Eryptose aus dem Blutkreislauf entfernt werden, entsteht Anämie. Später im Infektionsverlauf wurde zusätzlich eine intravaskuläre Lyse der roten Blutzellen beobachtet.

Die infektiöse Anämie beim Schwein ist besonders stark ausgeprägt wenn der Erregerbefall sehr hoch ist. Dies ist ein Hinweis darauf, dass *M. suis* einen direkten Einfluss auf die Hämolyse und damit die Anämie hat. Hämolyse kann durch starke Schäden und Deformationen an der Plasmamembran der Wirtszelle ausgelöst werden, die entstehen wenn *M. suis* an die Zelle adhäriert. Die Symptome der IAP könnten also durch Hemmung des Adhäsionsvorganges gelindert werden. Bisher ist erst ein Adhäsionsprotein von *M. suis* detailliert beschrieben. Hierbei handelt es sich um MSG1, ein Protein, das der Glyceraldehyde-3-phosphat dehydrogenase sehr ähnlich ist. Das Protein wurde als potentielle Vakzine getestet, konnte aber keine Protektion vermitteln. *In vitro* Experimente, die im Zusammenhang mit der vorliegenden Doktorarbeit durchgeführt wurden, zeigten, dass Aktin der roten Blutzellen ein Bindungspartner von MSG1 ist. Eine Bindung induziert Umlagerungen und strukturelle Veränderungen am Aktin, was zu der oben erwähnten fehlgeleiteten Immunantwort führen könnte.

3. Introduction

3.1 The genus Mycoplasma

3.1.1 Microbial properties

Mycoplasmas are bacteria lacking a cell wall. They are the smallest organisms (0.2 to 2 µm in diameter) known to be able to live autonomously. Mycoplasmas are pleomorphic and naturally resistant to antibiotics targeting cell wall synthesis. The genome sizes vary between 0.5 and 2.0 x 10^9 Da (0.5 to 2.0 kb) [1]. A characteristic for *Mycoplasma* is a low GC content of the DNA (23-46 mol %) [2]. Further, they use an alternate genetic code i.e. the *Mycoplasma* / *Spiroplasma* code (No. 4) that translates UGA, a common stop codon, into the amino acid tryptophan. Due to the reduced genome and the requirement for sterol for cell membrane rigidity most species are closely dependent on a host cell and are therefore difficult to cultivate *in vitro*.

3.1.2 Taxonomy

Bacteria lacking a cell wall are classified within the phylum *Tenericutes* (lat. tener – tender; cutis - skin) which comprises one class, the *Mollicutes* (lat. mollis – soft) [3]. The *Mollicutes* are further divided into five orders, one of which is referred to as the *Mycoplasmatales*. The single family within this order is called *Mycoplasmataceae* and harbours two genera, *Mycoplasma* and *Ureaplasma*. Based on the 16S rDNA sequences the genus *Mycoplasma* is assigned into three non taxonomical groups, i.e. pneumoniae, hominis and fermentans. The pneumoniae group divides into several clusters, one of which consists of haemotrophic mycoplasmas (HM), also called haemoplasmas [4].

3.1.3 Pathogenicity

Depending on the species, mycoplasmas exhibit commensalic, parasitic or saprophytic lifestyles. Pathogenic species isolated from vertebrates often cause inflammations in the respiratory tract (*M. pneumoniae*, *M. mycoides* subsp. *mycoides, M. gallisepticum*), in the genitourinary tract (*M. hominis*, *M. genitalium*) and in joints (*M. arthritidis*) [5-7]. Additionally, they can cause sepsis (e.g. *M. mycoides* subsp. *mycoides*, *M. pneumoniae, M. hominis)* [8-10].

Most pathogenic mycoplasmas express attachment proteins. *M. pneumoniae* for example developed a tip structure used for adherence and gliding motility on surfaces. Other mechanisms are toxins and mitogenic components (*M. neurolyticum, M. arthritidis*) [11, 12].

The bacteria often have to survive in inhospitable environments namely on cells exposed to a flow of body fluids. Therefore they developed mechanisms to induce morphologic and metabolic alterations in host cells to establish a suitable niche to avoid detection by the host's immune system. The interference with immune response often lead to development of clinical symptoms as inflammation and misled immune responses [13]. The ability of mycoplasmas to influence the immune system to their advantage allows for persistency and therefore favours development of chronic diseases. Thus, mycoplasmas attract more and more notice to medical research. As an example, *M. pneumoniae* was recently detected to be involved in the pathogenesis of adult rheumatoid arthritis, juvenile idiopathic arthritis, Crohn's disease and asthma [14].

3.1.4 Haemotrophic mycoplasmas and *M. suis*

HM live and replicate on red blood cells of their host and are not cultivable *in vitro*. In 1928 Schilling detected the presence of ring shaped structures on mouse erythrocytes and identified them as organisms named *Eperythrozoon coccoides* (Greek: "spherical animal on red blood cell") [15]. The existence of *E. wenyonii* in cattle and *E. ovis* in sheep and goats was reported in 1934 and 1943, respectively [16, 17]. In 1950 Splitter described a new species of erythrocyte-associated parasites in pigs, *Eperythrozoon suis* [18]. In the meantime similar pathogens have been described in cats (*Haemobartonella felis*) [19, 20], dogs (*H. canis*) [21] and rats (*H. muris*) [22]. Historically, the differentiation of the genus *Eperythrozoon* from *Haemobartonella* based on phaenotypic characteristics. In contrast to *Haemobartonella* cells *Eperythrozoon* members can appear ring shaped. Additionally, *Eperythrozoon* cells appeared as well free floating in the plasma whereas members of the genus *Haeobartonella* always were attached to the red blood cell. A species differentiation due to these characteristics was then considered as arbitrary [23]. Based on 16S rDNA sequencing, members of the genera *Eperythrozoon* and *Haemobartonella* were reclassified in 2001 to the genus *Mycoplasma* [4, 24, 25].

HM exhibit distinct species specificity and have been described to occur in several animal species, i.e. cats (*M. haemofelis, Candidatus* M*. haemominutum, Candidatus* M*. turicensis*) [26, 27], dogs (*M. haemocanis, Candidatus* M*. haematoparvum)* [28, 29], mice (*M. coccoides, M. haemomuris*) [4, 30], rats (*M. haemomuris*) [4], reindeers (*Candidatus* M. *haemotarandirangiferis*) [31], cattle (*M. wenyonii*) [32], sheep and goats (*M. ovis*) [33], opossums (*Candidatus* M. *haemodidelphis*) [28], llamas (*Candidatus* M. *haemolamae*) [28], squirrel monkeys (*Candidatus* M. *kahanei*) [34] as well as in pigs (*M. suis*) [4]. Further, the occurrence of HM in human beings was reported [35-37]. In one case, a HIV positive Brazilian patient was found to be infected with *M. felis*. The other two papers describe the situation in China. Between 1994 and 2007 12840 cases of human beings infected with haemotrophic mycoplasmas were registered. One study reports a *M. suis* prevalence of 35.3 % in the local population of Inner Mongolia. Among these population 57.0 % of pregnant women and all their newborn babies were tested positive.

HM are intimately associated with their host cell and are only separated from its membrane by an electron lucent zone of about 30 nm width. They are connected by fibril structures made of so far unknown proteins [38]. Recently, *M. suis* has been described to be able for cell invasion [39] (Figure1). Figure 2 shows *Mycoplasma suis* cells on red blood cells.

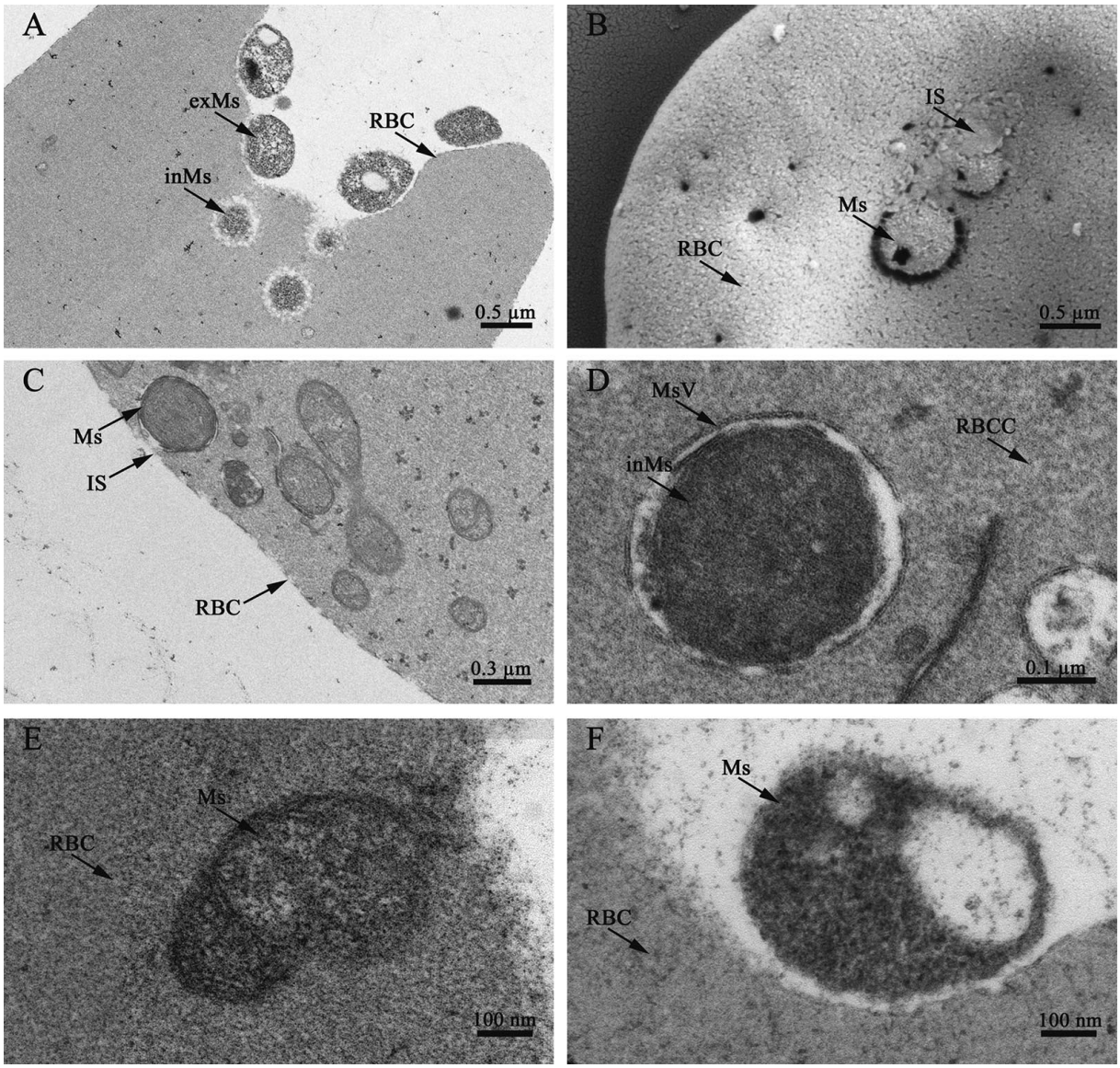

Figure 1 Invasion of porcine erythrocytes by *M. suis* 08/07 shown by scanning electron microscopy (B) and transmission electron microscopy (A and C to F). During invasion *M. suis* 08/07 is located in deep invaginations of the RBC membrane (A). As invasion progresses, the erythrocyte membrane conforms to the shape of the bacterial cells (A and C), and newly formed membrane material covers the surface of the bacteria (B and C). As a result, invading mycoplasmas are located within an intraerythrocytic vacuole (D). Intracellular forms that are free in the RBC cytoplasm (E) have a shape and size similar to the shape and size of extracellular attached *M. suis* cells (F). exMs, extracellular *M. suis*; inMs, intracellular *M. suis*; IS, invasion scar; Ms, *M. suis*; MsV, *M. suis* vacuole; RBCC, RBC cytoplasm. Figure from [39].

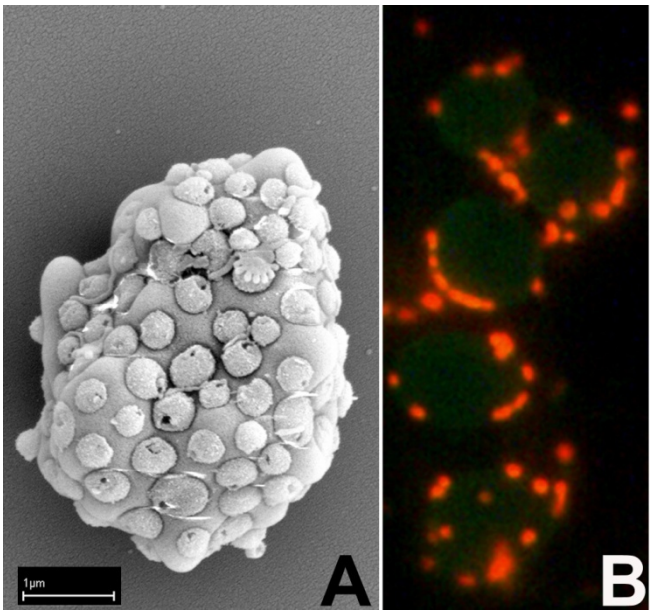

Figure 2 Close association of *M. suis* cells with porcine red blood cells. A: scanning electron micrograph showing a red blood cell infected with *M. suis*; B: Acridine orange stained blood smear.

The polymorphic bacterium is approximately 200 nm in diameter and harbours a circular genome of 0.7 to 0.8 x 10^9 Da (0.7 to 0.8 kb) that has not been sequenced so far [40]. Due to the *in vitro* unculturability the bacteria are propagated in the splenectomised and therefore immunocompromised pig leading to special challenges. In all experiments researchers have to deal with the heterogenic porcine background in purifications of bacteria. Further, the time point of sampling is often difficult to plan. The time course of disease depends on the age of the infected pig and the quality of the *M. suis* inoculum. Despite this fact, eight immunogenic proteins were described by our research group [41]. Two of them were characterised in detail. MSG1, a protein similar to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is surface localised and involved in adhesion [42]. HspA1, a DnaK–like protein, was found by serological proteome analysis and shown to be expressed at the surface of *M. suis* [43]. Both proteins were expressed in *E. coli* to perform functional studies. MSG1 expressing *E. coli* clones were shown to attach to red blood cells *in vitro* and the purified, recombinant protein exhibited glycolytic activity. The recombinant HspA1 protein showed ATP activity. Both purified proteins were recognised by convalescent sera of *M. suis* infected animals and therefore were used to establish an ELISA test for serological diagnostics [44].

An additional protein i.e. the inorganic pyrophosphatase (PPase) was found by *M. suis* library screening [45]. The activity of the *M. suis* enzyme was strongly dependent on Mg^{2+} and significantly lower in the presence of Mn^{2+} and Zn^{2+}. Addition of Ca^{2+} and EDTA inhibited the *M. suis* sPPase activity. These characteristics confirmed the affiliation of the *M. suis* PPase to family I soluble PPases. The highest activity was determined at pH 9.0. In *M. suis* the sPPase builds tetramers of 80 kDa which were detected by convalescent sera from experimentally *M. suis* infected pigs.

3.2 Anaemia

The word anaemia is of Greek origin and stands for “lack of blood”. Anaemia is diagnosed as diminution of haemoglobin concentration, haematocrit or erythrocyte number below the reference values [46]. There are four principle mechanisms for anaemia to occur, i.e. (1) deficiency of red blood cell production, (2) increased rate of red blood cell destruction or degradation, (3) loss of red blood cells by bleeding and (4) circulatory disturbances due to accumulation in enlarged spleen [47]. Patients suffering from anaemia often appear pale, feel weak and tired and have problems to concentrate. Severe anaemia leads to hypoxia and therefore to dyspnoea [46].

3.2.1 Autoimmune haemolytic anaemia

Human autoimmune haemolytic anaemia (AIHA) shows an incidence of 1:45000 in western countries [48]. Patients produce antibodies directed against their own red blood cells [49]. In warm AIHA said antibodies are of IgG isotype and reactive at 37 °C. About 50 percent of warm AIHA are idiopathic, e.g. no underlying disease is diagnosed. In contrast, secondary warm AIHA is associated with other illnesses such as lymphoproliferative diseases, systemic lupus erythematosus (SLE), intoxications or viral infections (e.g. Eppstein Barr Virus, Rubella Virus) [50]. Most IgG antibodies recognise the Rhesus complex, which also carriers blood group determinants [51]. The expression of autoreactive IgG antibodies is T-cell dependent requiring the presence of helper T-cells to induce the class switch from IgM to IgG. An imbalance between protecting regulatory T-cells and autoaggressive T_{H1}-cells due to altered expression of cryptic epitopes could be responsible for loss of tolerance to host proteins [52].

Additionally, agglutinating IgM antibodies targeting carbohydrate structures (I/i epitope) at temperatures below 37 °C, occur. Three forms of cold AIHA have been

described, namely spontaneous (seldom, higher risk due to age), idiopathic and secondary. Occasionally, the latter develop upon infection with *Mycoplasma pneumoniae* [53]. Nothing is known about a physiological role of cold reactive antibodies although very small concentrations of cold agglutinins are present in sera of most healthy human beings [54].

3.2.2 Anaemia in pigs

In swine, not many triggering factors for anaemia are described. Most common are iron deficiency and infectious anaemia of pigs (IAP) caused by infection with *M. suis.* Further reasons for anaemia in pigs include umbilical haemorrhagia, intoxications, parasitism and infection with circovirus.

Acute clinical signs of IAP are accompanied by bacteraemia and include haemolytic anaemia, hypoglycaemia, acrocyanosis, icterus, fever and finally death [55]. Tetracycline treatment saves the pigs from death but bacteria are not eliminated from the host's body. The disease converts to a chronic state characterised by low-grade anaemia, higher susceptibility to other infections, poor reproductive performance of sows and growth retardation of piglets. Chronic signs are mainly responsible for economic losses caused by IAP. Stress can lead to recrudescence of the disease showing above mentioned acute clinical signs but every further "clinical attack" is characterised by less severe clinical signs. Usually, pigs infected experimentally with *M. suis* survive from the fourth attack without medical treatment which gives evidence for the generation of a protective immune response. Chronically infected animals are *M. suis* carriers.

IAP is prevalent worldwide and of considerable economical significance for the pig industry. A prevalence of 30 percent is reported for the U.S.A. and one of 10 to 30 percent for China [56, 57]. In Germany, 12 percent of 6 to 8 week old piglets are infected [58]. Similar prevalences are expected to occur in Switzerland.

M. suis cells are passed on to other pigs by transfer of infected blood. This often happens iatrogenically. Further, the blood sucking arthropods *Stomoxys calcitrans* (stable fly) and *Aedes aegypti* have been discussed to play a role as vectors [59].

One characteristic for anaemia is a low blood haemoglobin (Hb) concentration. The reference value for pigs lies between 108 to 148 g/L blood depending on their race and age [60]. Pigs suffering from IAP show life threatening anaemia. The lowest Hb concentration observed during our studies was 33 g/L. Pigs are pale, icteric and show laboured respiration.

The knowledge about the pathogenicity of the anaemia in context with *M. suis* infection is sparse. Hoelzle et al. detected the presence of autoreactive antibodies of the IgG isotype during the acute phase [41]. Additionally, cold agglutinins occur during the chronic phase. In analogy to human cold reactive antibodies induced upon infection with *M. pneumoniae* it is assumed that they target glycosylated proteins on the red blood cell membrane [61, 62]. So far, the epitopes have not been characterised. Refer to Figure 3 for illustration.

Figure 3 Visualisation of cold agglutinins. A: Pig with cyanosis at the ear tips. Red blood cells are agglutinated in the peripheral capillaries where body temperature is lower than 37 °C. B: EDTA anticoagulated blood incubated at 4 °C and C: at 37 °C. The agglutination is reversible.

3.3 Eryptosis

The normal life span of a human red blood cell is about 120 d. After this time, the cell is removed from circulation by macrophage uptake. Due to lack of nucleus and mitochondria it was assumed that red blood cells were not able for controlled cell death. However, erythrocyte senescence is characterised by conformational changes as cell shrinkage, plasma membrane microvesiculation, change in shape from discocyte to a sphaerocyte, cytoskeleton alterations associated with protein (spectrin) degradation and loss of plasma membrane phospholipid asymmetry leading to externalization of phosphatidyl-serine (PS, Figure 4) [63, 64]. In 2001, two independent groups described that mature erythrocytes can undergo apoptosis-like cell death [65, 66]. In analogy to apoptosis this process was referred to as eryptosis [67].

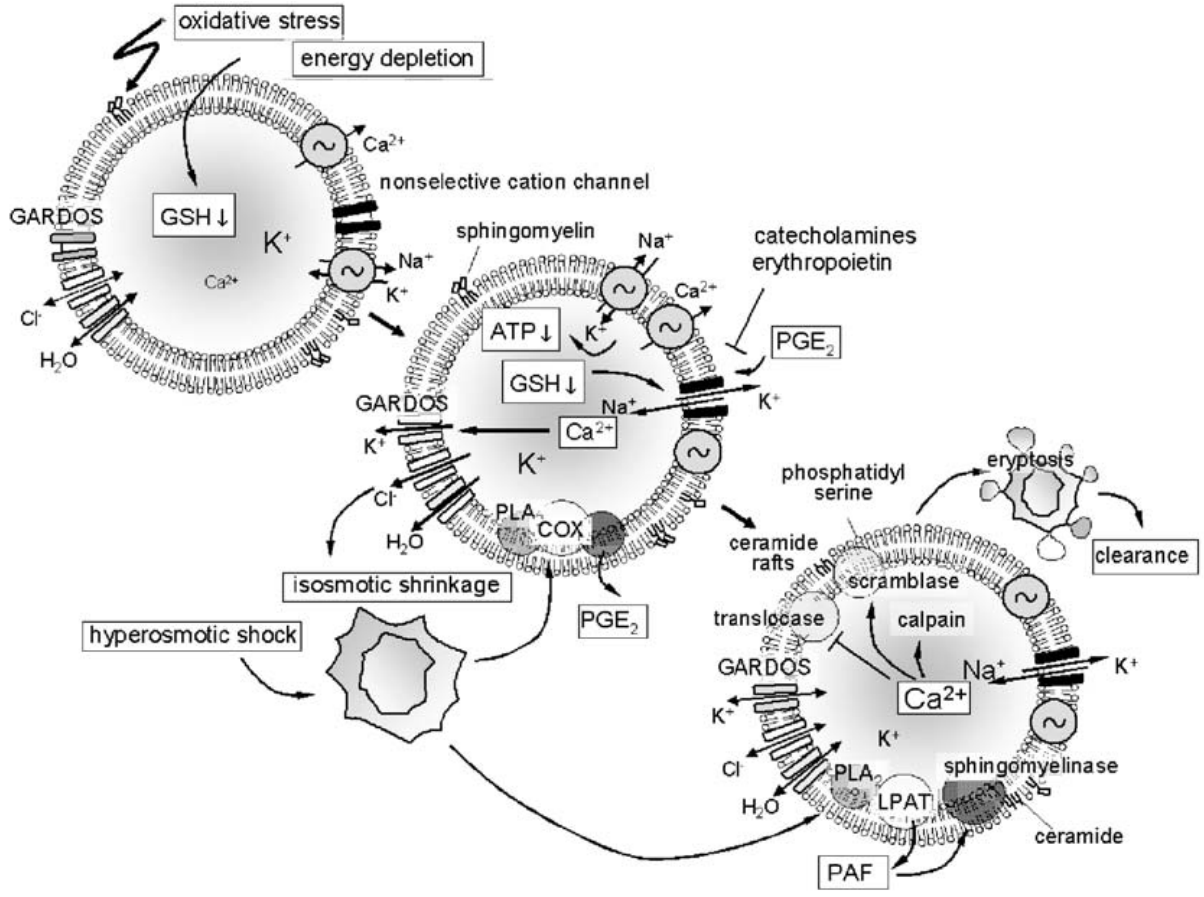

Figure 4 Mechanisms and signaling pathways involved in eryptosis i.e. cellular shrinkage, phosphatidylserine exposure and activation of calpains. COX, cyclooxygenase; GSH, reduced glutathione; GARDOS, potassium channel; LPAT, lyso-PAF acetyl transferase; NSC, nonselective cation channel; PAF, platelet-activating factor; PGE_2, prostaglandin E_2; PLA_2, phospholipase A_2. From [68].

Similar mechanisms occur in malaria patients. By invading red blood cells plasmodia try to evade the immune system of the host. They induce host expression of cation channels in erythrocyte membranes to ensure availability of Na^+ and Ca^{2+} ions and the possibility of waste disposal. This leads to accumulation of Ca^{2+} ions in the cells and consequently to induction of eryptosis. By this defence mechanism, stressed, pathogen-invaded cells are removed from the circulation system without haemolysis [69]. Further, the occurrence of eryptosis in sepsis patients has been described [70]. Red blood cells obtained from healthy donors underwent eryptosis after incubation in plasma from these patients. The authors identified sphingomyelinase-induced accumulation of intraerythrocytic ceramide as triggering factor for PS exposure.

4. Objectives and Contents

This thesis deals with the elucidation of pathogenesis of the severe infectious anaemia (IAP) of *M. suis* infected pigs. A combination of at least three mechanisms is involved: (I) autoimmune processes, (II) controlled cell death of red blood cells (eryptosis) and (III) lysis due to damage by the close interaction of *M. suis* and the red blood cell. In this thesis, the analysis was approached considering changes on both sides, i.e. on the host (swine) and on the pathogen (*M. suis*).

4.1 Analysis of host's response

In response to infection immunological processes are induced to fight invaders. Normally, specific antibodies are produced to protect the host. Against that, immune responses are misregulated and directed against host structures in *M. suis* infected pigs. In this thesis, analysis of such autoreactive antibodies and identification of autoantigens were a central topic.

Stressed or damaged body cells often undergo apoptosis to prevent inflammation. Erythrocytes are able for controlled cell death by a process termed eryptosis. In this thesis the occurrence of eryptosis was evaluated to better understand host defence mechanisms and to resolve its the impact on the pathogenesis of the anaemia.

4.2 Analysis of pathogenicity factors

Lysis of erythrocytes due to direct damage by attachment and invasion processes of *M. suis* is quite evident. Observable deformations, invaginations and invasion scarves are generated on the infected red blood cells. Therefore, detection and analysis of pathogenic factors as proteins used for attachment, invasion or antigenic variation of *M. suis* were a main issue of this doctoral thesis.

5. Results and Methods

This chapter gives an overview about the results obtained during this Ph.D. thesis and the methods used to achieve these results. The methods used in experiments that resulted in publications or manuscripts are described in chapter 8.

5.1 Analysis of host's response

5.1.1 Autoreactive antibodies

Two types of autoreactive antibodies targeting erythrocyte proteins occur during a *M. suis* infection, i.e. IgG warm and IgM cold antibodies. Aim of this thesis was to characterise these antibodies.

IgG warm autoreactive antibodies

Porcine actin was identified as target of autoreactive warm IgG antibodies by ELISA and immunoblots. Actin is recognised mainly during the time points of clinical attacks. Details are described in **manuscript 8.1**.

IgM cold autoreactive antibodies

Cold reactive IgM antibodies are upregulated during the chronic state of the disease, i.e. when the pathogen load is decreased. In analogy to human IgM cold agglutinins induced by *M. pneumoniae* infection it is most likely that glycosylated proteins are targets of cold agglutinins occurring during IAP. Therefore, glycoproteins were purified by ether elution [71] (Figure 5). A monoclonal antibody targeting porcine Glycophorin A (GlpA), one of the major sialoglycoproteins of the erythrocyte membrane, was used to control the presence of GlpA and therefore the quality of the purification. Sera containing IgM cold agglutinins were tested of reactivity with these glycoprotein purifications by western blot, but no reaction was observed. Therefore, it is unlikely that GlpA is a target of cold reactive IgM antibodies induced by *M. suis* infection.

We decided to enlarge the search for autoreactive antigens to non-glycosylated proteins. For this, western blots with whole cell preparations of porcine erythrocytes, lymphocytes, murine macrophages and Buffalo Green Monkey cells were performed. More bands were reactive on blots if incubated at 4 °C than at 37 °C. Corresponding proteins will be identified by mass spectrometry in a planned master thesis project.

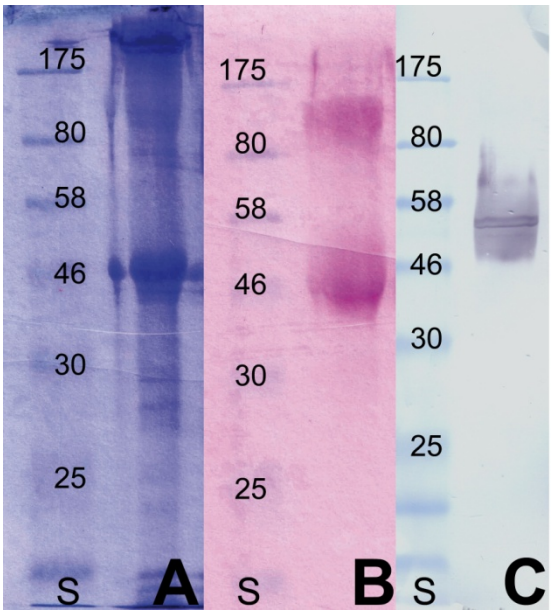

Figure 5 Purification of glycosylated proteins from porcine erythrocyte membranes. Polyacrylamide gels stained with coomassie R-250 for general protein detection (A) and with Schiff-Base for glycoprotein detection (B); C: western blot showing porcine GlpA. S: Protein mass standard in kDa.

Methods used for IgM cold reactive antibody characterisation

Purification of cold agglutinins

To purify cold reactive IgM antibodies, blood samples were put to 37 °C immediately after withdrawal to prevent binding of cold agglutinins to the red blood cells. During the subsequent clotting process, cold reactive IgM antibodies remained in the soluble serum phase. After centrifugation at 2000 x g for 10 min at room temperature, IgM containing serum samples were stored in aliquots at -20 °C until use.

Visualisation of cold reactive IgM antibodies

Animals were observed for acrocyanosis. Blood samples taken at these time points were evaluated for reversible cold agglutination of erythrocytes. For this, EDTA blood test tubes were incubated at 4°C for 30 min and photographed. Then, samples were exposed to a temperature of 37 °C for 15 min and examined for solubilisation of agglutinates.

Western blot

Sera were used for western blots (as described elsewhere [41]). Briefly, cell preparations of Buffalo Green Monkey (BGM) cells, mouse macrophages (J774), porcine erythrocytes and lymphocytes as well as *M. suis* purifications and actin preparations were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Free binding positions were blocked by

2 % (w/v) skim milk in Tris buffered saline (blocking buffer). Next steps (incubation with test sera (1:100 in blocking buffer) and with a secondary antibody (1:500 in blocking buffer) targeting porcine IgM conjugated to horse-radish-peroxidase (HRP)) were done in parallel at 4°C and 37 °C over night and for 2 h, respectively. Bound antibodies were detected by providing H_2O_2 as substrate and adding 4-Chloro-1-naphtol for chromogenic development.

5.1.2 Eryptosis

Programmed cell death of red blood cells (eryptosis) as response to infection with *M. suis* was evaluated by fluorescence-activated cell sorting (FACS) on a FACS Calibur instrument (Becton Dickinson). Preliminary results indicated that severity of anaemia and occurrence of eryptosis correlated. A study to further characterise this finding was designed and performed. Animals were splenectomised and infected with *M. suis*. Eryptosis occurrence and haematological parameters were recorded over the time course of infection. Serum and plasma of an *M. suis* infected pig was shown to induce eryptosis *in vitro*. Detailed description of methods and obtained results are given in **manuscript 8.2**.

5.2 Analysis of pathogenicity factors

5.2.1 Adhesion protein MSG1

For a pathogen, a prerequisite for infection of a host cell is the capability for attachment. We identified an immunogenic protein of *M. suis* that is involved in the attachment process. The GAPDH homologous protein was recombinantly expressed in *E. coli*. This protein exhibited glycolytic activity and was expressed at the surface. It was referred to as MSG1 (*M. suis* glycolytic protein 1). The full length paper is presented in **chapter 8.3**.

The intimate interaction of *M. suis* with the red blood cell leads to severe deformations and damage at the host cell membrane. If the impairments are strong enough cell lysis and consequently anaemia occurs. Knowledge about the interaction partner(s) of MSG1 on the red blood cell would help to develop therapy or immunisation strategies based on inhibition of attachment. In this thesis receptor studies were performed and porcine actin was identified as potential receptor protein for MSG1. This finding was confirmed by inhibition ELISAs and surface plasmon resonance.

By ligand blots two proteins of the red blood cells that are bound by MSG1 were detected and identified as porcine catalase and cytoskeletal actin (β-actin), respectively. A representative blot is shown in Figure 6.
To show specificity of binding, the interaction was competitively inhibited by adding actin during incubation. This analysis was done by ELISA. As indicated in Figure 7, a significant inhibition was observed.
Additional proof for a specific interaction of MSG1 with actin was obtained by surface plasmon resonance (SPR). Two experiments were performed. Once, porcine α-actin was the bait and MSG1 the analyte. The second experiment was conducted vice-versa (α-actin was used as analyte and MSG1 as ligand). A tendency for specific interaction could be observed for both experiments. The binding was very weak due to low concentrated protein purifications.

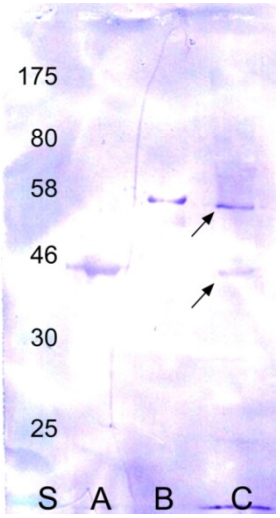

Figure 6 Interaction of MSG1 with porcine proteins on a ligand blot. A: porcine α-actin, B: bovine catalase, C: porcine ECL, S: protein mass standard in kDa. Arrows show two protein bands that were identified as porcine catalase and β-actin, respectively.

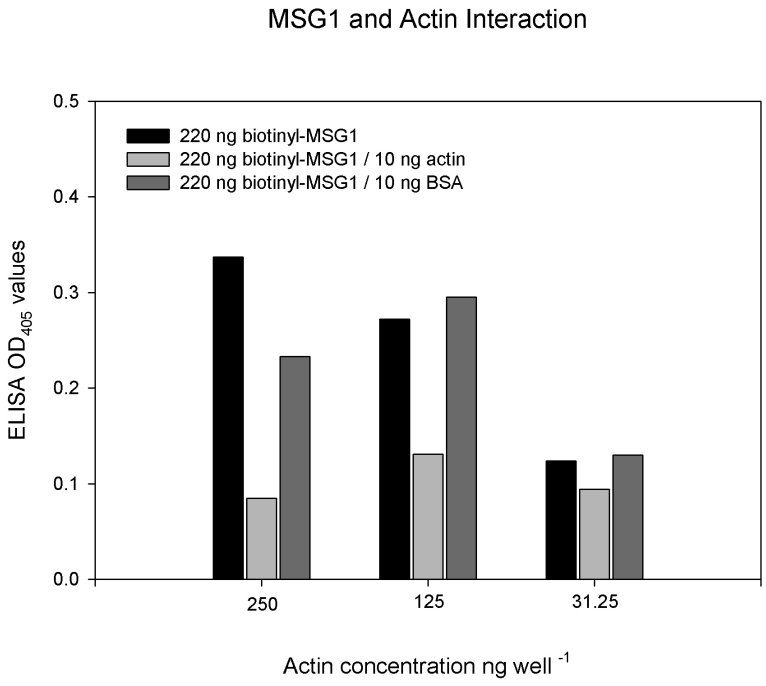

Figure 7. Inhibition ELISA showing the specificity of binding between MSG1 and porcine actin. The binding of biotinylated MSG1 to actin is competitively inhibited by incubation in presence of actin. BSA does not inhibit the binding.

Methods to analyse interaction of MSG1 with host proteins

Ligand blot

Purified erythrocytes of healthy pigs were lysed by ice cold hypotonic phosphate buffer (5 mM Na_2PO_4, 1 mM EDTA, pH 7.6) for 5 minutes on ice and centrifuged at 30000 x g for 15 min at 4 °C. This white pellet was resuspended in lysis buffer and the centrifugation step repeated until the pellet appeared white. The white pellet was resuspended in cold 1xPBS to a concentration of 1 mg/ml, stored at -80 °C until further use and designated as erythrocyte lysate (ECL).

ECL was separated by SDS PAGE and blotted to a nitrocellulose membrane. The 6xHis tagged recombinant MSG1 protein was purified by nickel affinity chromatography and biotinylated by the biotin labeling kit (Roche) according to the manufacturer's recommendations. 100 mg of biotinylated MSG1 was incubated with the previously blocked membrane (1% FKS in Tris buffered saline; 1xTBS: 50 mM Tris base, 150 mM NaCl, pH 7.6). After stringent washing with 1xTBS containing 0.05 % Tween 20, bound protein was detected with peroxidase labelled streptavidin. H_2O_2 was used as substrate and 4-Chloro-1-naphtol as chromogenic agent.

The proteins of interest were identified by matrix assisted laser desorption ionisation-time of flight coupled to tandem mass spectrometry (MALDI-TOF MS/MS). The analysis was performed by Toplab, Martinsried, Germany.

Inhibition ELISA

100 ng of porcine α-actin were coated to individual wells of 96 well microtiter plates. After blocking the free binding positions (1% FKS in 1xPBS, 30 min, room temperature) incubation was performed with different concentrations of biotinylated MSG1 for 2 h. For inhibition, 10 ng of α-actin were added per well during incubation. As a negative control, 10 ng of BSA were added.

Surface plasmon resonance (SPR)

SPR allows the analysis of binding properties between two proteins. A bait protein (ligand) is coupled to a matrix (gold) and surface plasmons (electromagnetic waves propagating in parallel to the gold interface) are allowed to pass it until a steady state is reached. Then the prey (analyte) is added. Upon binding, the surface and therefore the wavelength of the surface plasmon, changes. The reflection angle of a light beam directed to the surface plasmon is as well changed and can be recorded (response units = RU). The analyte then is removed from the ligand. From the signals association (v_{on}) and dissociation rates (v_{off}) are determined. The dissociation constant then can be calculated by $K_d=v_{off}/v_{on}$.

Figure 8 illustrates the principle of the SPR method. The experiments were done with Stefan Schauer in the functional genomics centre, Zurich, with the Biacore T100 instrument (GE Healthcare).

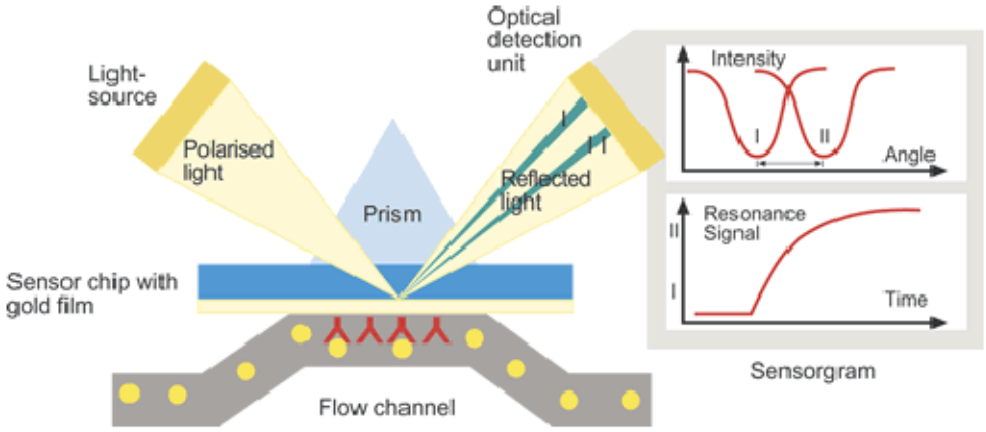

Figure 8 Principle of Surface Plasmon Resonance (SPR). A light beam hits a surface plasmon. The detected emission angle changes if the surface plasmon changes by encountering a different surface structure. A typical association and dissociation signal of a SPR binding assay between ligand and analyte is shown in the sensorgram.
(http://www.rci.rutgers.edu/~longhu/Biacore/MolecularInteractionAnalysis.html).

5.2.2 Immunogenic adhesion protein MSG1 as vaccine candidate

MSG1 was evaluated for the potential of acting as protective immunogen. Unfortunately, no significant protection was observed. During this experiment, some animals showed clinical signs that were more severe than usual. These animals had to be put out of their misery by euthanasia. It was not clear, whether immunisation with MSG1 worsened symptoms instead of being protective. For details refer to **publication 8.4**.

5.2.3 Proteomic analysis to detect further *M. suis* proteins

Of particular interest were proteins that could be involved in pathogenesis of anaemia. We decided to use liquid chromatography coupled to mass spectrometry (LC-MS). Proteins are roughly separated by conventional SDS-PAGE and peptides of interest are further separated by liquid chromatography and identified by mass spectrometry. Compared to separation by means of two dimensional gel electrophoreses the method requires lower amounts of protein.

To identify proteins a database containing all published protein sequences of species classified as *Mollicutes* was created and the identified peptides were compared to these sequences. Using the uniref100 database of the European Bioinformatics Institute, peptides of porcine origin could be recognised and were neglected. In Figure 9 a representative gel is shown and Table 1 gives an overview of detected proteins. The genome of *M. suis* has not been sequenced so far, therefore, only the sequences of MSG1 and HspA1 could be considered in the database. In all *M. suis* containing samples the two *M. suis* proteins were clearly identified. For other proteins we were dependent on cross-species identification. Mycoplasmas generally show low homologies between different species, both on DNA and on protein level. Consequently, the proteins could not be identified definitely. However, 21 of found proteins were confirmed on genomic level in context with a concomitant Ph.D. study that deals with sequencing of a shot gun *M. suis* library (Table 1).

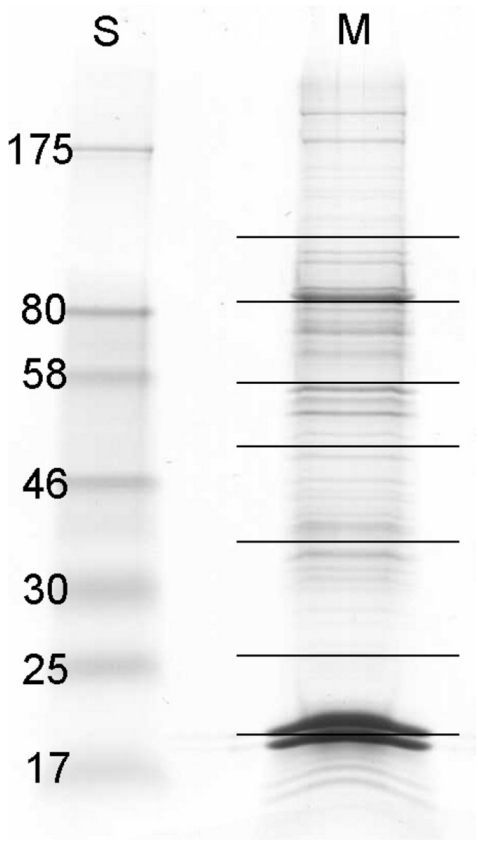

Figure 9 Coomassie stained gel showing separation of *M. suis* proteins for LC-MS sample preparation. S: Molecular weight standard in kDa, M: *M. suis* purification. The gel was fragmented according to the horizontal bars prior to digestion with trypsine.

Table 1 Proteins identified by LC-MS/MS of species of the taxonomic Class *Mollicutes*. The two *M. suis* proteins are shaded. By screening a shot gun library of *M. suis* some genes were sequenced. The right column indicates if protein identification could be confirmed (√) on genomic level.

Protein	Species	*M. suis*
30S ribosomal protein S2	*M. gallisepticum*	
3-dehydroquinate synthetase	*Acholeplasma laidlawii*	
60 kDa chaperonin	*M. genitalium*	
ABC transporter, ATP binding protein	*M. penetrans*	
ABC transporter, permease protein	*M. agalactiae*	√
ABC-type amino acid transport system, permease component	Onion yellows phytoplasma	√
Appendage associated protein precursor	*Anaplasma marginale*	
Arginyl-tRNA synthetase	*M. agalactiae*	
Aspartyl-tRNA synthetase	*M. hyopneumoniae*	√
ATP dependent protease La	*M. agalactiae*, Aster yellows witches'-broom phytoplasma	√
ATP synthase subunit B	*M. penetrans, M. pulmonis, M. mobile, M. synoviae, M. hyopneumoniae, Mesoplasma florum*	√
Beta-ketoacyl-(acyl carrier protein) synthetase	*Acholeplasma laidlawii*	
Cell division protein ftsZ	*M. genitalium*	√
Chaperone protein dnaJ	*M. mycoides subsp. mycoides* SC	
Chaperone protein dnaK	*M. pulmonis, M. gallisepticum, M. hyopneumoniae, Anaplasma marginale, Mesoplasma florum*	√
Chromosome segregation ATPase	*Mesoplasma florum*	
Cytadherence high molecular weight protein 1	*M. genitalium*	
Cytadherence high molecular weight protein 2	*M. pneumoniae*	
Cytoskeletal protein	*M. penetrans*	
degV family protein	*M. capricolum* subsp. capricolum ATCC 27343	
Dihydrolipoamide dehydrogenase	*M. synoviae, M. agalactiae*	
DNA directed RNA polymerase subunit beta	*M. penetrans, M. gallisepticum, M. capricolum* subsp. *capricolum, U. parvum, M. pulmonis, M. genitalium, M. hyopneumoniae, M. mycoides* subsp. *mycoides* SC*, Spiroplasma citri*	√
DNA polymerase III alpha subunit	*M. synoviae*	
DNA polymerase III subunits gamma and tau	*M. hyopneumoniae*	
DNA processing protein smf	*M. mobile*	
DnaA	*M. gallisepticum*	
DppD	*M. gallisepticum*	
Elongation factor G	*M. genitalium, M. gallisepticum, M. agalactiae, M. penetrans, M. mobile*	√
Elongation factor Tu	*M. hominis, Mesoplasma florum,* Apple	

Protein	Species	*M. suis*
	proliferation phytoplasma	
Endoglucanase	*M. synoviae*	
Fructose-bisphosphate aldolase	*M. synoviae, M. pulmonis*	
Glucose-6-phosphate isomerase	*M. agalactiae*, Aster yellows witches'-broom phytoplasma	
Glyceraldehyde-3-phosphate dehydrogenase	*M. pneumonia,* Onion yellows phytoplasma	√
Glycerol-3-phosphate dehydrogenase	*M. mobile*	
HspA1	*M. suis*	√
HtpA	*Mycoplasma* phage MAV-1	
Hystidiyl-tRNA synthetase	*M. mobile*	√
Integral membrane protein	*M. penetrans, Mesoplasma florum*	
Leucyl-tRNA synthetase	*M. penetrans, M. capriculum* subsp. *capricolum*	√
Lipoate-protein ligase A	Onion yellows phytoplasma	
Lipoprotein	*M. capricolum* subsp. *capricolum*	
Lysophospholipase	*M. mycoides* subsp. *mycoides SC*	
Membrane-anchores GGDEF domain protein	*Acholeplasma laidlawii*	
Membrane-bound ATP-dependent metalloprotease	*Acholeplasma laidlawii*	
Methionyl-tRNA synthetase	*M. penetrans*	√
MSG1, g1 adhesin	*M. suis*	√
Mg^{2+} ion transporter	*U. parvum*	√
Na^{+} ABC transporter, ATP binding component	*M. mycoides* subsp. *mycoides* SC	√
NADH oxidase	*Mesoplasma florum*	
Phenylalanin-tRNA synthetase beta chain	*M. pulmonis*	
Phosphatidate cytidylyltransferase	*M. agalactiae*	
Phosphoenolpyruvate-protein phosphotransferase enzymeI	*M. mobile, Acholeplasma laidlawii, M. mycoides* subsp. *mycoides* SC, *M. hyopneumoniae*	√
Phosphoglyceromutase	*Mesoplasma florum*	√
Prolipoprotein	*M. mycoides* supsp. mycoides SC	
Prolipoprotein B	*M. mycoides* subsp. mycoides SC	
Pseudogene of transposase (C-terminal)	*M. agalactiae*	
Pseudouridine synthase B	*M. mobile*	
Phase-variable hemagglutinin, putative	*M. synoviae*	
Pyruvate kinase	*M. hyopneumoniae*	
RecA	*M. pneumoniae*	√
Ribosome binding factor A	*M. genitalium, M. pneumoniae*	
Ribosome recycling factor	*M. genitalium*	
Topoisomerase 1	*M. gallisepticum*	
Topoisomerase IV subunit B	*M. hyopneumoniae*	
Translation inititation factor IF-2	Aster yellows witches'-broom phytoplasma	
Type I restriction enzyme r protein	*M. mobile*	

Methods used for proteome analysis

Sample preparation

Freshly drawn red blood cells with attached *M. suis* cells were washed 3 times in 1xPBS to remove plasma proteins. The red blood cells were diluted in a 1:1 ratio (v/v) in 1xPBS and incubated for two hours at 37 °C. By this procedure the *M. suis* cells detach from their host cells. Then, red blood cells were removed from the samples by low speed centrifugation (200 x g, 5 min). The supernatant was mixed with 2x SDS PAGE sample buffer (0.15 M Tris, pH 6.8, 1 % SDS, 30 % Glycerol, 15 % β-mercaptoethanol, 0.002 % bromophenol blue) in a ratio of 1:1 (v/v) and boiled for 5 min. Samples were loaded on 10 % polyacrylamid gels and proteins were electrophoretically separated according to their mass. Gels were incubated for 1 h in fixation solution (40 % ethanol, 10 % acetic acid) and stained with colloidal coomassie G-250 over night. The staining solution consisted of 10 % w/v $(NH_4)_2SO_4$, 20 % w/v ortho-phosphoric acid, 25 % v/v methanol, 6 % w/v coomassie brilliant blue G-250.

Tryptic digest

The gel was sliced to 8 pieces, distained (incubation in 50 % methanol in 100mM NH_4HCO_3, pH 8.0 for 3 h at 37 °C) and each gel piece was digested with trypsine (0.001 % trypsine in 5 mM Tris, pH 8.0, 6 h at 30 °C). Peptides were extracted from the gel by means of 0.1 % and 10 % (v/v) formic acid (FA) and concentrated by an Eppendorf Speedvac centrifuge. Peptides were resolved in a buffer containing 5 % (v/v) Acetonitrile (ACN) and 0.1 % (v/v) FA. Samples then were desalted using C18-ZipTips (Millipore).

Peptide identification

Analysis of peptides was done by liquid chromatography coupled to mass spectrometry (LC-MS/MS) in the functional genomics centre (FGCZ) by Bernd Roschitzki as described in [72]. Briefly, samples were resuspended in 5% v/v ACN, 0.2% v/v FA (Buffer A) and loaded onto a reverse-phase capillary column (RP C18, 75 mm_8 cm; 200A, AQ; Bischoff GmbH) using a fully automated nanoflow LC system consisting of a PAL autosampler (CTC Analytics AG) and a binary Rheos 2000 pump (Thermo Scientific). Liquid chromatography was performed using a 90-min gradient by means of Buffer A and B (80% v/v ACN, 0.2% v/v FA). Peptides were eluted with the following linear gradient: 0–3 min, 0% solvent B; 3–53 min, 0–50% solvent B; 53–63 min, 50–100% B followed by 100% B for 4 min and 100% A for 23 min. The LC system was directly coupled to an ion trap mass spectrometer, equipped with a nanospray ionization source (LTQ ESI Trap, ThermoScientific). Each MS full scan was followed by the acquisition of up to three data-dependent MS/MS spectra of the three most intense peaks. Parent masses used for MS/MS were dynamically excluded for 0.5 min.

6. Discussion

6.1 Analysis of host´s response

Infectious anaemia of pigs (IAP) is of economic significance due to a prevalence of 10 to 30 percent in Western countries. Animals show growth retardation, are susceptible to other infections and sows show reduced fertility. A main clinical sign in acutely ill pigs is the severe anaemia that occurs upon stress. Knowledge about the pathogenesis of this anaemia is rare due to drawbacks in research. These include the unculturability of *M. suis* and the dependency on the pig as propagation vessel which leads to background of porcine DNA or protein in all samples. It is evident, that several mechanisms collude in generation of anaemia in a complex way. Among these are autoreactive processes, programmed cell death and direct red blood cell lysis by interaction with *M. suis*.

There are two types of autoreactive antibodies involved in *M. suis* infection. IgM cold agglutinins occur during later stages of infection leading to acrocyanosis and consequently necrosis of harmed tissues. Target structures are not identified. In analogy to human cold agglutinin disease induced by *M. pneumoniae* it is assumed that glycoproteins on the erythrocyte membrane are recognised [61, 62]. In this dissertation, the reactivity of these antibodies with a glycoprotein purification of red blood cells was tested but no reactivity was observed. The quality of the glycoprotein purification was controlled with a monoclonal antibody targeting Glycophorin A (GlpA), a protein that is present in high amounts on membranes of red blood cells. Probably, glycoproteins of lower abundance were too low concentrated to be detected in the glycoprotein purifications used. However, some reactive bands were obtained in western blots with whole cell preparations conducted at 4 °C. Cold agglutinins are responsible for the milder anaemia developing in the chronic phase of the disease.

In contrast, there are autoreactive antibodies of the IgG isotype [41]. They are upregulated during the acute phase of IAP. In this thesis, actin was identified as a target. In eukaryotes there are three types of actin, i.e. α-actin, β-actin and γ-actin. The autoreactive IgG antibodies recognise α-actin and β-actin; however, there is a preference for β-actin. This actin form is present in cytoskeletons of eukaryotic cells, including the red blood cell.

An upregulation of autoreactive antibodies is the consequence of loss of tolerance to self antigens. Actin, that usually is cryptic, gets accessible for immune cells by interaction of *M. suis* with the red blood cell. Upon attachment, the plasma membrane is damaged and gets permeable for antibodies. The finding, that MSG1 and actin interact *in vitro* supports this theory. *M. suis* maybe is able to dissolve the plasma membrane by enzymatic activity. Further, there is evidence that actin is rearranged by invasion of *M. suis [39]*. Changed host proteins are no more recognised as self which leads to activation of an adaptive specific immune response directed against host actin.

Actin recognising B-cells are present in healthy organisms and are necessary for tissue homeostasis [73]. Due to an unspecific mitogenic stimulus induced by *M. suis* infection, B-cells recognising actin could be stimulated by a thymus independent mechanism (T_i-immune reaction) [62]. The fact that mycoplasmas can activate B-cells without regard to antigen specificity was described to occur for several *Mycoplasma* species, e.g. *M. pulmonis*, *M. arginini*, *M. fermentans* or *M. pneumoniae* [74-77]. To further elucidate the role of this unspecific blastogenesis in context with *M. suis* infection further studies are required.

A widespread mechanism for induction of autoreactivity in context with infections is molecular mimicry. This means that a pathogen shares an epitope with a host protein. To find potential epitopes special algorithms, i.e epitope finder programs have been developed. For swine lymphocyte antigens (SLA) the NetMHCpan program is available [78]. By feeding the sequences of porcine β-actin and *M. suis* MSG1 into the program, a potentially cross-reactive epitope was found. The peptides LTLKYPIEH and RTLKYYISL derived from β-actin and MSG1, respectively, are theoretically presented by the SLA-2*0201 molecule to circulating T-cells. Due to degeneracy of both, the T-cell receptor and the MHC peptide-binding motifs, the identity of 55 % of these two proteins theoretically is enough for cross-reactivity [79, 80]. Interestingly, clinical signs are only observed in the blood circulation system. If the actin present in muscle cells would be targeted, corresponding clinical signs would be expected. This substantiates the theory that *M. suis* has to be locally present to induce autoreactive antibody formation.

So far, *M. suis* cells as well have been exclusively found inside the circulatory system. It is not clear why the bacterium selected the red blood cell as its habitat. On one hand, this cell type is small, does not harbour many nutrients, does not

synthesise any proteins, is not stationary and has a limited life span of about 120 days. But on the other hand, red blood cells are the only body cells lacking MHC I molecules. This allows intracellular pathogens to evade the immune response mediated by cytotoxic T-cells. Additionally, the erythrocyte harbours high concentrations of iron bound to haemoglobin and of glucose. Glucose levels were shown to be negatively correlated to *M. suis* loads [81]. An obvious explanation was that bacteria consume the glucose directly. It was reported that red blood cells infected with Plasmodia exhibited a 40 to 100 times higher glucose consumption rate compared to uninfected cells [82]. For *M. suis* infection of porcine red blood cells it remains to be elucidated whether *M. suis*, the infected red blood cell or both together are responsible for glucose level diminution in the blood.

In any case, the integrity of the infected red blood cell is challenged. Upon stress a suicide program is started and the single cell sacrifices itself in favour of the whole organism. This process, referred to as eryptosis, can therefore be defined as defence mechanism against intracellular pathogens. If high numbers of cells get infected, eryptosis can be responsible for triggering anaemia. In blood samples of *M. suis* infected animals higher amounts of PS exposing red blood cells were detected than in samples of healthy pigs. A negative correlation of eryptotic cell number to haematocrit levels was recorded during the growth phase of *M. suis* cells indicating a causal interrelation. In this phase, the anaemia seems to be induced by programmed cell death of erythrocytes. During maximal bacteraemia the red blood cells were not stained anymore with PKH-26, a membrane dye that exclusively stains cells with intact cell membranes, i.e. healthy and eryptotic cells. It is evident that another mechanism of cell death (intravascular haemolysis due to autoreactive antibodies or direct damage by the bacterium) took over. At this time-point, when loads of up to 4000 *M. suis* cells per red blood cell were recorded, the mechanism of eryptosis to protect the organism simply was exhausted. However, proof for eryptotic activity even at this time point is given. Incubation of red blood cells from healthy pigs with serum or plasma taken during bacteraemia led to breakdown of membrane asymmetry *in vitro*. The serum and plasma samples contained *M. suis* cells. Therefore, it has to be elucidated whether the triggering factor is *M. suis* or a soluble compound in the plasma.

6.2 Analysis of pathogenicity factors

Due to their small genome mycoplasmas are not able to synthesise all essential molecules. This leads to a close association with and a dependency on their host cell. By this intimate contact, changes on the host cell are inevitable. This could, as mentioned above, lead to actin rearrangements. From another intraerythrocytic organism namely *Plasmodium falciparum* it is known that it induces the expression of ion channels in the plasma membrane of the red blood cell for acquisition of Ca^{2+} and Na^{+}-ions and disposal of waste products [82, 83]. An elevation of Ca^{2+} influx leads to breakdown of membrane asymmetry by exposure of phosphatidyl-serine (PS) residues to the outer leaflet [84]. The same happens in normal erythrocyte senescence. The exposed PS of an old red blood cell are recognised by macrophages and the cell is safely removed from the circulation system without causing inflammation [64]. At a first glance, eryptosis is unfavourable for an intracellular pathogen. However, exposed PS leads to attachment to vascular endothelial cells and therefore offers the opportunity of settledness [85].

M. suis infection of red blood cells leads to severe deformations. To invade the cell, bacteria manipulate the membrane. If this is done excessively, the membrane integrity is broken and the cell is lysed leading to anaemia. To get knowledge about mechanisms causing cell lysis it is important to identify pathogenicity factors as attachment proteins and proteolytic enzymes. One attachment protein, the MSG1, was identified, purified and characterised [42]. The immunogenic protein is analogous to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and expressed at the surface of *M. suis*. *In vitro* it interacts with porcine actin. Cytoskeletal actin lies inside the plasma-membrane of cells and therefore is not directly accessible. Thus, *M. suis* must have additional tools to protrude the membrane. To find such pathogenicity factors an attempt of analysing the proteome of *M. suis* was carried out. This was hampered due to unavailability of genome data of *M. suis*. Therefore, the peptides were compared to protein sequence data of other members of the taxonomical class of the *Mollicutes*. Within the genus *Mycoplasma* homologies in genome and proteome data are low. For instance, the identity of the genes encoding glucose-6-phosphate isomerase of *M. penetrans* and *M. synoviae* is only 46.1 %. On protein level, the identity score is 39.1 % and the similarity score 61.4 %. Due to the dependency of cross-species identification the scores in our analyses were very low (between 20 and 70 %) and the identifications therefore not secure. Proteomics

specialists expect an identity score of > 95 % for a positive identification of a protein. However, some promising preliminary data were obtained and genome sequencing data of shot gun library clones endorsed the protein identifications in 21 cases. Generally, *Mycoplasma* proteins exhibit multiple functions due to the small genome sizes. For example, proteins involved in the carbohydrate metabolism have been described to act as virulence factors [42, 86]. Eight of the identified proteins in Table 1, including MSG1 of *M. suis*, are involved in sugar metabolism. Additionally, there were two cytadherence proteins and a putative phasevariable hemagglutinin. These proteins are involved in pathogenicity. It was shown that loss of cytadherence protein function in *M. pneumoniae* led to non-adherent growth and loss of cytotoxicity [87]. Hemagglutinis are lipoproteins that cause red blood cells to agglutinate. An example is VlhA of *M. synoviae*, a protein used for attachment to the erythrocyte [88]. Loss of hemagglutinin function leads to apathogenicity [89]. In order to confirm these identifications and to find additional *M. suis* proteins, the identified peptides will be re-analysed as soon as the genome data are available.

7. Milestones and Outlook

The pathogenesis of the anaemia induced by *M. suis* infection is due to a complex interplay of several mechanisms. During this thesis some aspects were clarified, i.e. the occurrence of autoreactive antibodies targeting porcine actin and the upregulation of eryptotic processes. Additionally, evidence for several pathogenicity factors was found. For the attachment protein MSG1 a potential receptor on the red blood cell was identified, i.e. porcine actin. Figure 10 gives an overview of the involved mechanisms.

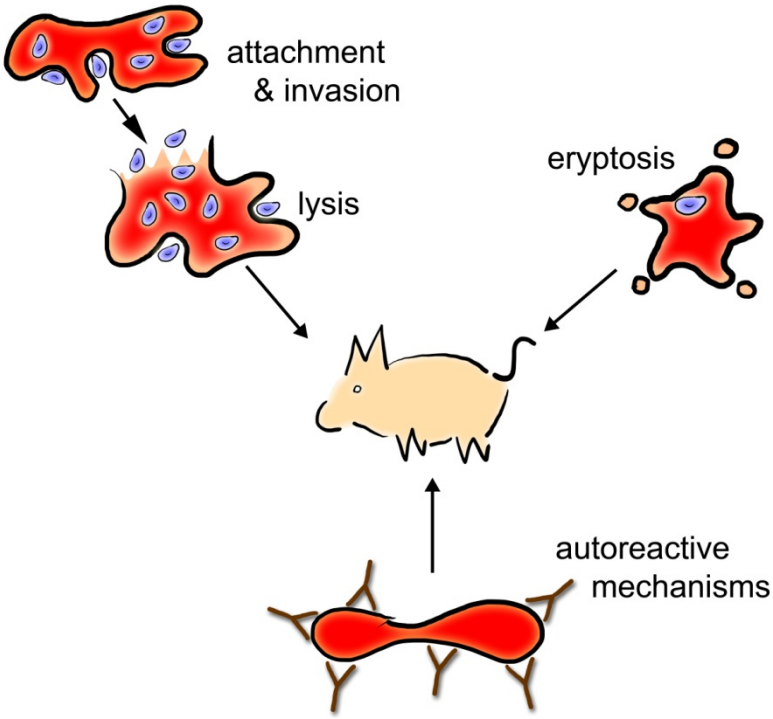

Figure 10 Overview about the pathogenesis of the haemolytic anaemia in pigs due to *M. suis* infection. A complex interplay of several factors originating from the host as well as from the pathogen is taking place. The pale pig suffers from anaemia that is induced by autoreactive mechanisms, eryptosis and direct haemolysis due to damages resulting from attachment and invasion processes of mycoplasmas.

The experiments performed during this dissertation elucidate aspects of the anaemia pathogenesis and are the basis for clarification of further questions.

- The host immune system plays a crucial role for the outcome of the disease. Clarification of the involved mechanisms therefore was and still is a main topic in our research group. This includes the detection of target epitopes of cold agglutinins and the elucidation of the mechanisms involved in autoreactivity. Mycoplasmas are able to interfere with the host's immune system e.g. by providing mitogenic stimuli on unrelated B-cells. Thereby, the specific immune response against *M. suis* is masked due to an excess of antibodies that recognise non-mycoplasmic structures.

- A further strategy to evade the host's immune response is antigenic variation. To explore the ability of *M. suis* for antigenic variation the expression profile during the time course of infection is observed by proteome analysis. In parallel, changes in expression of pathogenicity factors are recorded.

- Immune evasion is achieved by hiding in the red blood cells that lack MHC I molecules. Infected cells therefore are not recognised by the immune system. The only mechanism left to safely remove the infected cell is programmed cell death. In fact, the majority of red blood cells are infected during bacteraemia. Clearance of these cells therefore aggravates the anaemia. Additionally, eryptotic cells exhibit PS residues at their surface mediating the ability for attachment to endothelial cells which leads to circulatory disturbances. Thus, future experiments deal with clarification of the triggering factors for eryptosis.

- *In vitro* experiments indicate that MSG1 binds porcine actin. All experiments considering actin and MSG1 interaction were performed with muscular actin (α–actin) of the pig. Red blood cells as well as other body cells predominantly contain the cytoskeletal actin form (β-actin). However, this protein was commercially not available. Therefore, it was expressed in *E. coli*. Additionally, fragments of β-actin are produced to identify the actual binding regions of actin.

- The obtained proteome data indicate the presence of several pathogenicity factors of *M. suis*. Due to lack of genome data the identification scores were in general too low for acceptance in the scientific community. Currently, a genome sequencing project is running. *M. suis* DNA was separated from porcine DNA by selective digestion of the porcine DNA and sequenced by the 454 method developed by Roche. The obtained sequences are currently annotated. With these data at hand additional peptides will be identified and the proteomic results obtained in this thesis will be confirmed and can be published.

8. Manuscripts and Publications

In this section, a submitted manuscript, a manuscript prior to submission and two peer reviewed publications including results associated with this Ph.D. thesis are presented. Own contributions are described for each part.

8.1 Antibodies to actin in autoimmune haemolytic anaemia

Kathrin M. Felder, Katharina Hoelzle, Karl Heinritzi, Mathias Ritzmann, and Ludwig E. Hoelzle

Submitted to *BMC Veterinary Research*

In this manuscript we describe for the first time actin as autoantigen in connection with autoimmune haemolytic anaemia.

I cloned and expressed porcine β-actin, performed analysis of sera by ELISA and western blot and wrote the manuscript.

Antibodies to actin in autoimmune haemolytic anaemia

Kathrin M. Felder[a], Katharina Hoelzle[a], Karl Heinritzi[b], Mathias Ritzmann[c], and Ludwig E. Hoelzle[a*]

[a]Institute of Veterinary Bacteriology, Vetsuisse Faculty, University of Zurich, Switzerland
[b]Clinic for Swine, Ludwig-Maximilians-University Munich, Munich, Germany
[c]Clinic for Swine, University for Veterinary Medicine, Vienna, Austria

Abstract

Background: In Autoimmune haemolytic anaemia (AIHA) autoreactive antibodies directed against red blood cells are upregulated leading to erythrocyte cell death. *M. suis* infections in pigs (IAP) induce AIHA of both the warm and the cold type. Aim of this study was to identify target autoantigens of warm autoreactive IgG antibodies. For this, sera from experimentally *M. suis* infected pigs were screened for autoreactivity.

Results: In sera of 95 % of all tested animals actin-reactive antibodies were found. The reactivity was shown to be species specific, i.e. reactivity with porcine actin was significantly higher than with rabbit-actin. Sera of animals previously immunized with the adhesion protein MSG1 of *M. suis* showed reactivity with actin prior to infection with *M. suis* indicating molecular mimicry to be involved in specific autoreactive mechanism. A potentially cross reactive epitope could be detected.

Conclusions: This is the first report of autoreactive anti-actin antibodies involved in pathogenesis of autoimmune haemolytic anaemia.

* Corresponding author: PD Dr. L. E. Hoelzle, Winterthurerstr. 270, CH- 8057 Zurich; Phone: 004144658613; Fax: 004144658912

Background

Human autoimmune haemolytic anaemia (AIHA) shows an incidence of 1:45000 per year in western countries [1, 2]. 70 % of patients suffer from warm, 15 % from cold AIHA. Warm AIHA is characterised by IgG antibodies binding their target epitope at body temperature [3]. The target membrane antigen is usually within the Rhesus system, although often the antibody specificity cannot be defined [4]. In cold AIHA complement activating autoreactive IgM antibodies bind their antigen, usually the I/i epitope on red blood cells, below body temperature and are strongly agglutinating at 4 °C [5, 6]. Cold AIHA is divided into acute and chronic forms. Acute forms often occur from between two to three weeks after an infection with mycoplasmas, e.g. *M. pneumoniae*, Epstein-Barr-Virus or Rubella virus [6]. About 50 % of warm cases are classified as idiopathic, meaning that no obvious underlying disorder is known. For secondary warm AIHA several causes have been identified, i.e. Non-Hodgkin-Lymphoma, systemic lupus erythematosus, drugs and viral infections. In 7 % of AIHA both types of antibodies occur; this is referred to as mixed AIHA. Sokol and co-workers report only one case of secondary mixed AIHA due to *Mycoplasma* infection [2].

Haemotrophic mycoplasmas infect red blood cells of several mammalian hosts [7, 8]. Generally, they show distinct host specificity. However, reports on human beings infected by haemotrophic mycoplasmas, i.e. *Mycoplasma haemofelis* and *Mycoplasma suis* have been published [9, 10].

M. suis causes infectious anaemia of pigs (IAP) [7]. Within this disease, the presence of mixed AIHA was found. IgM cold agglutinins targeting glycoproteins on the red blood cells were observed [11, 12]. These cold agglutinins occur about 4 weeks after experimental infection with *M. suis*. During the acute stage of IAP (clinical attack), acute anaemia, hypoglycaemia and icteroanaemia accompanied with high mortality occur. It is assumed that the severe anaemia observed in pigs suffering from acute clinical signs is due to a combination of direct damage of red blood cells by *M. suis* attachment and invasion as well as an upregulation of cold and warm autoreactive antibodies directed against red blood cell components. In previous studies, these autoreactive warm IgG antibodies were shown to overwhelm the specific immune response to *M. suis* [13].

In this study, sera of experimentally *M. suis* infected pigs were screened for the presence of warm IgG antibodies by testing their reactivity with blood preparations of healthy pigs. The detection and characterisation of host proteins that served as autoantigens was a main issue. Earlier studies, i.e. serological proteome analysis [14] rendered some evidence for actin to be a target protein of autoreactive antibodies detected during the acute state of the disease. This was observed in one- as well as in two-dimensional immunoblots. Therefore, sera tested in this study were additionally screened for reactivity with muscular (α-) and cytoskeletal (β-) actin of the pig.

Results

Study design and screening of porcine sera for autoreactive antibodies

A collection of sera obtained from experimentally *M. suis* infected pigs was available from earlier studies [13, 15]. Sera were divided into two groups according to their history. Group 1 sera (36 sera) derived from pigs (n=13) that were vaccinated with MSG1 prior to splenectomy and challenge with *M. suis* infection. Due to the known main occurrence of autoreactive IgG antibodies during clinical attacks [13] sera used for the present study were drawn at following time points: I. 21 days post immunisation and 14 days prior to *M. suis* infection, II. during the first clinical attack (on average 10 days after infection), III. 14 days apart, IV. during the second clinical attack (on average 6 weeks post infection). Due to death of animals less sera could be analysed at time points II (n=11), III (n=6) and IV (n=6). Group 2 sera originated from 10 pigs that were not immunised prior to splenectomy and *M. suis* infection. It consisted of 40 sera. For this study sera taken prior to infection (I), during the first clinical attack (II), in between the first and the second clinical attack (III) and during the second clinical attack (IV) were considered. In total, 76 sera (36 of group1 and 40 of group 2) were screened by ELISA for reactivity with IgG depleted antigen preparations from healthy pigs (neg Ag). OD values were normalised to the negative control. The cut-off value was determined to be 0.226 (three times standard deviation of negative controls).

Figure 1a gives an overview of screening results. At time point I, prior to splenectomy and infection, 84.6 % of sera of group 1 (immunised with MSG1) and no sera of group 2 showed autoreactivity. During the first clinical attack a shift in antibody response could be observed. Reactivity of group 1 sera decreased to 45.5 %. 60.0 % of group 2 sera reacted with neg Ag. Between two clinical attacks 83.3 % of sera of immunised pigs showed autoreactivity, sera of group 2 down regulated antibodies to neg Ag completely. During the second clinical attack 100.0 % of sera in group 1 and 80.0 % of sera in group 2 were autoreactive. Reactivities between groups differed significantly at every time point. *P* values were determined to be ≤ 0.006, 0.010, 0.004 and 0.043 for I, II, III and IV, respectively. Only for one animal out of 23 no autoreactive antibodies were detected in sera taken at any time point. Figure 1b shows occurrence of autoreactive antibodies during time course of infection for one animal of each group. The vaccinated animal representing group 1 already produced

autoreactive antibodies after immunisation with MSG1 and prior to infection and splenectomy. Autoreactive antibodies remained on a similar level for time points II, III and IV. The animal selected to represent group 2 did not show autoreactive antibodies before infection, upregulated antibodies to neg Ag at timepoint II (1st clinical attack), down regulated them below the cut-off value between clinical attacks and again produced autoreactive antibodies during the 2nd clinical attack (IV).

Reactivity with porcine α-actin

In order to identify autoantigens, the found autoreactive sera (n=36) were further tested with IgG depleted erythrocyte lysates (ECL) and with porcine α-actin. In earlier studies hints for actin as a potential target of warm autoreactive antibodies were found (one- and two dimensional immunoblots) [14]. In 32 sera (88.9 %) antibodies reactive with both preparations were detected. Linear regression for neg Ag with ECL and α-actin revealed R^2 values of 0.732 and 0.744, respectively (Fig. 2).
The actin reactivity was shown to be species specific, i.e. a significant lower reactivity was observed with rabbit α-actin than with porcine α-actin. Mean OD values at λ = 405 nm were 0.408 ± 0.136 and 0.793 ± 0.185 for rabbit- and for porcine α-actin, respectively.

Reactivity with porcine β-actin

The erythrocyte contains cytoskeletal actin (β-actin). This actin form is highly homologous but not identical to the muscular actin (α-actin). Since β-actin was not commercially available the gene was synthetically produced and adapted to the *E. coli* codon usage. The protein was expressed, purified and used for the experiments (Fig. 3). Sera reactive with α-actin were tested further with β-actin. All these sera were positive with both actin forms. For group 1, the MSG1 immunised animals, a strikingly stronger reactivity with β-actin than with α-actin was observed ($P \leq 0.0002$). No significant difference was seen in sera of group 2 animals ($P \leq 0.2034$).

Isotypes of autoreactive antibodies to actin

In warm autoimmune haemolytic anaemia IgG_1 and IgG_3 antibodies predominate [16]. These antibodies are recognised preferably by macrophages. To gain insight

into autoreactive mechanisms occurring during a *M. suis* infection, secondary antibodies to porcine IgG_1 and IgG_2 were used for evaluation of subtypes of actin reactive antibodies of group 1. Secondary antibodies to porcine IgG_3 were not available. The ratios IgG_1/IgG_2 and IgG_2/IgG_1 were calculated to be 1.280 ± 1.796 and 2.727 ± 1.925, respectively ($P \leq 0.018$).

Search for shared epitopes between MSG1 and actin

Reactivity with actin was observed in MSG1 vaccinated immunocompetent animals previous to splenectomy and *M. suis* infection. To support the hypothesis of molecular mimicry, the reactivity of a rabbit hyperimmune serum against recombinant MSG1 with actin and vice-versa, i.e. reactivity with hyperimmune serum against porcine actin with MSG1 was evaluated. Such cross-reactivity was observed (Fig. 4). Therefore, the protein sequences of porcine actin as well as of MSG1 were used as input in an epitope finder program. The program allows identifying epitopes potentially presented by SLA molecules to B-cells with high probability. SLA-2*0201 would present the peptide LTLKYPIEH derived from actin and the peptide RTLKYYISL derived from MSG1. Both peptides are 9 amino acids in length and share a sequence identity of 55 %. This peptide is identical in both actin forms.

Discussion

Autoimmune haemolytic anaemia (AIHA) is an autoimmune disorder characterised by autoreactive antibodies directed against an individual's own red blood cells (RBCs) leading to enhanced clearance of RBCs by Fc receptor (FcR)-mediated phagocytosis [17]. About 50 percent of human warm AIHA cases are idiopathic [18], i.e. no underlying disorder is known. Amongst others, conceivable causes of AIHA are infectious diseases. There are cases in which AIHA has been associated with viral, bacterial or parasitic infectious agents [3, 5]. *M. pneumoniae* is often involved in cold AIHA.

In *M. suis* infections autoreactive antibodies are of central importance for the pathogenesis, i.e. in producing anaemia [11, 12]. Beyond cold reactive antibodies of the isotype IgM targeting so far unidentified erythrocyte carbohydrates, warm autoreactive antibodies of the isotype IgG upregulated during the acute phase of an experimentally induced *M. suis* infection have been described by our group [13]. However, detailed characteristics of potential antigens targeted by these autoreactive IgGs remained undescribed. Two dimensional immunoblot analysis revealed only one porcine protein, i.e. actin being potentially recognised by these antibodies [13, 14]. Actin is known to play a role in autoimmune hepatitis type 1 (AIH-1) where F-actin reacting antibodies are characteristic [19]. To our knowledge, this is the first report and evidence for autoreactive antibodies directed against actin in warm AIHA.

In particular, characterising these autoantigenic structures and its mechanisms is an essential basis for understanding the pathogenesis of warm AIHA when associated with *M. suis* infections, and in general. Typically, induction of autoreactive antibody production is caused by a misguided upregulation of naturally occurring B-cells specific for self antigens, the occurrence of altered self antigens, the appearance of previously cryptic antigens, and a loss of tolerance to self antigens due to molecular mimicry.

In healthy humans as well as in animals antibodies recognizing cytoskeletal components as actin, spectrin or band 3 are necessary to maintain tissue homeostasis [20]. Therefore, B-cell clones specific for actin are continually patrolling. A misregulation due to *M. suis* infection could lead to excessive thymus independent proliferation of these B-cell clones [12]. Other *Mycoplasma* species, i.e. *M. pneumoniae*, have been demonstrated to be a polyclonal B-cell activator of mouse

splenocytes [21]. Further, Zachary and Smith have described such misdirected immune responses to occur in *M. suis* infections [12]. Whether an uncontrolled proliferation of B-cells is the reason for autoreactive IgG molecules targeting actin is a topic of future studies. Actually, actin targeting IgG antibodies are upregulated during the first clinical attack when *M. suis* is present in high numbers. This renders evidence for the hypothesis of a direct mitogenic stimulus derived from *M. suis* leading to unspecific B cell stimulation.

A further explanation for the development of autoreactive antibodies is that due to damage of red blood cells hidden cytoskeletal proteins become accessible to circulating antibodies, be interpreted as non-self and elicit an immune response. It has been hypothesised that autoimmune epitopes on red blood cells may be a result of contacts with proteolytic enzymes [22]. Electron microscopic studies revealed a close association of *M. suis* cells with the erythrocyte as well as the occurrence of striking membrane deformations on the host cell [23, 24]. Considering loads of up to 4000 *M. suis* cells per erythrocyte it is quite evident that the cytoskeleton is modified by attachment and invasion. Whether active actin remodelling is taking place is topic of current investigation in our laboratory.

The last explanation for the induction of autoreactive antibodies directed against host's actin in *M. suis* infections is a phenomenon called molecular mimicry. In this process, a peptide derived from a pathogen and presented by MHC II must be capable of activating a self reactive helper Tcell and, therefore, stimulating specific antibody producing B-cells.

Remarkably, in sera obtained from pigs that were immunised with MSG1 auto-actin-antibodies already were detectable after immunisation but prior to splenectomy and challenge with *M. suis*. These findings strongly support the hypothesis that MSG1 is involved in inducing autoimmunity by molecular mimicry. Evidence for cross-reactivity between MSG1 and actin was gained in a heterologous system. To test whether actin and *M. suis* actually share potential cross reactive epitopes, the NetMHCpan algorithm [25] was used revealing a positive result. The porcine SLA-2*0201 (swine lymphocyte antigen) would strongly bind and therefore present the peptide LTLKYPIEH derived from porcine actin as well as RTLKYYISL derived from MSG1 to the same circulating T-cells. The identity between these peptides was calculated to be 55 % which is enough for cross-reactivity. Degeneracy in both the TCR and MHC peptide-binding motifs reduces the sequence-specific requirement to only a few

crucial residues [26-28]. It remains to be elucidated by systematic *in vitro* stimulation assays whether these peptides actually play a role in pathogenesis of AIHA due to *M. suis* infection.

In RBCs as in many other cell types β-actin is present. Only in muscle cells α-actin is predominant. If the identified strongly β-actin binding antibodies would encounter their target ubiquitously, not only RBCs would be affected. There is evidence that lymphocytes and epithelial cells are harmed during *M. suis* infection (own unpublished observations). Cytoskeletal proteins in cells with intact plasma membranes are cryptic for antibodies. However, if the cell got harmed by *M. suis* interaction, β-actin would become accessible. Notably, clinical signs expected by autoreactive processes targeting the muscular actin form (α-actin) have not yet been observed.

In autoimmune diseases including AIHA misdirected IgG_1 and IgG_3 antibodies play an important role due to efficient binding to FcReceptorIII (FcRIII) molecules on macrophages [16, 29]. Interestingly, the antibodies that predominantly recognised actin were rather of the subtype IgG_2. Immune complexes with this subtype activate the alternative pathway of complement cascade and are important in defence against encapsulated bacteria [30, 31]. However, the consequences of autoreactive IgG_2 upregulation during *M. suis* infection remain unclear. Unfortunately, no statement about the presence of IgG_3 antibodies and their role in *M. suis* infection can be given since secondary antibodies for this porcine subtype were not available.

Conclusions

Overall, we record that autoreactive IgG antibodies upregulated during the infectious anaemia of the pig induced by *M. suis* recognise actin. These autoreactive antibodies obviously are involved in the pathogenesis of the severe anaemia observed during the infection. Interplay of several mechanisms, i.e. a blastogenic response due to unspecific lymphocyte proliferation, direct damage of red blood cells and molecular mimicry are assumed to take place. To our knowledge, this is the first report of autoreactive actin antibodies in context with AIHA. Human idiopathic haemolytic anaemias do not show a very high incidence but patients suffer from severe symptoms. Haemotrophic mycoplasmas have not been cultivated *in vitro* and, therefore, are not detected in routine diagnostics. What if these unknown bacteria were to play a role? Infections with haemotrophic mycoplasmas of immune compromised human beings have been reported [9, 10]. As Sokol and co-workers stated in a review of 1985, pathogenesis of AIHA is very complex. They recommended that one retained an open mind and did not become too fervent a supporter of any particular theory [32]. In our opinion, the experimentally *M. suis* infected splenectomised pig represents a useful model for investigations in AIHA due to controllable inducible symptoms.

Methods

Preparation of screening antigens

To screen for autoreactive antibodies blood from healthy pigs was prepared accordingly to *M. suis* antigen preparations as described in previous publications [33, 34] with slight modifications. Briefly, blood cells from *M. suis* negative pigs were sedimented by centrifugation at 300xg for 15 min at room temperature (RT). Plasma and buffy coat were discarded. The erythrocytes were suspended in phosphate-buffered saline (1xPBS; Biochrom Ag, Berlin, Germany) containing 0.15 % Tween20 and 3 % EDTA, and incubated 20 min at RT with shaking. Debris and erythrocytes were removed by low-speed centrifugation (500xg, 20 min, RT). Supernatant was centrifuged at 25000xg for 120 min at 4 °C. The resulting pellet was resuspended in sterile 1xPBS at a concentration of 1 mg/ml. The preparation was IgG and albumin depleted using ProteoExtract® Albumin/IgG Removal Kit, Maxi (Calbiochem, Merck, Geneva, Switzerland) according to the manufacturer's recommendations and stored in 100 µl aliquots at -80 °C until further use. The preparations were referred to as negative Antigen (neg Ag).

Sera reactive with neg Ag were further tested with IgG depleted erythrocyte lysate (ECL), and with several actin preparations. ECL was prepared by hypotonic lysis of erythrocytes obtained from a healthy pig. Red blood cells were washed three times with 1xPBS (500xg, 15 min, RT) and resuspended in ice cold lysis buffer (5 mM Na_2PO_4, 1 mM EDTA, pH 7.6, Sigma) in a ratio of 1:1 (v/v). After incubation on ice for 5 min ghosts (remainings of lysed cells) were pelleted (30000xg, 15 min, 4 °C). This procedure was repeated until the pellet turned white. The white pellet was washed in 1xPBS, homogenised by ultrasonication on ice and IgG depleted. Concentration was set to 1 mg/ml with 1xPBS and stored in 1 ml aliquots at -80 °C until further use.

Muscular actin (α-actin) of pig and rabbit was obtained from Sigma, Buchs, Switzerland. Cytoskeletal actin of the pig (β-actin) was not commercially available and therefore produced recombinantly (see 2.3).

Screening of sera by ELISA

Sera were obtained from previous studies using a splenectomized pig model for the experimental *M. suis* infections [15]. Sera were divided into two groups: Group 1 consisted of sera of animals that were immunised with a recombinant immunogenic surface protein of *M. suis* (MSG1) [15] prior to splenectomy and *M. suis* infection. Sera in group 2 were from splenectomised, *M. suis* infected pigs without immunisation [13].

Porcine sera were screened for autoreactivity by ELISA as described previously [35]. Briefly, 100 ng of IgG depleted neg Ag were coated to individual wells of microtiter plates. Sera obtained from pigs used in previous studies [13, 15] were tested in duplicates. Sera were drawn prior to *M. suis* infection, after immunisation in group 1 (I), during first clinical attacks (II), between first and second clinical attack (III), and during the second clinical attack (IV). Sera reactive with neg Ag were further tested for reactivity with ECL, α-actin of pigs as well as of rabbits, and with porcine β-actin. Protein concentration was 100 ng / well. OD values (λ = 405 nm) were determined and normalised to a negative control (pool of 10 sera from healthy pigs). Cut-off values were set at three times the standard deviation of negative controls.

To determine subtypes of autoreactive antibodies monoclonal antibodies targeting porcine IgG_1 and IgG_2 (Prionics, Schlieren, Switzerland) were used 1:1000 in PBS/0.05 % Tween20. Horse-radish-peroxidase labelled antibodies to mouse IgG (Sigma) diluted 1:5000 in PBS/0.05 % Tween20 were used for detection.

Cloning and expression of porcine cytoskeletal actin

Cytoskeletal actin (β-actin) of the pig was commercially not available. Based on the encoding mRNA sequence (Genbank:AY550069), the gene was *de novo* synthesised and optimised for *E. coli* codon usage by Eurofins (Martinsried, Germany). For cloning, specific recognition sites for endonucleases were introduced (*Xho*I at the 5' end and *Hind*III at the 3' end). For expression, the gene ligated into the expression vector pBadMycHisA (Invitrogen) using T4 ligase obtained from Roche, Basel, Switzerland (overnight, 14 °C). The insert containing vector was transformed into *E. coli* LMG194 (Invitrogen). *E. coli* transformants were grown at 37 °C, expression of the recombinant porcine β-actin was induced by addition of 0.02 % arabinose at OD_{600} = 0.4. After incubation of the cells for additional 4 h at 37 °C, the 6xHis-tagged

protein was purified by nickel affinity chromatography (GE Healthcare, Glattbrugg, Switzerland) according to the manufacturer's recommendations. To solve the protein from inclusion bodies, 8M Urea was used for purification. The recombinant protein was further purified by electro elution out of 10 % polyacrylamide gels using the electro-eluter model 422 (Biorad, Reinach, Switzerland). The eluted protein was precipitated by adding 3 volumes of ice cold acetone and incubation over night at -20 °C. The protein was harvested by centrifugation (10000xg, 90 min, 4 °C), allowed to dry, resuspended in 1xPBS, and stored in aliquots of 1mg/ml at -80 °C.

Production of hyperimmune sera

One milligram of protein (recombinant MSG1 [15], recombinant porcine β-actin as well as porcine α-actin (Sigma, Buchs, Switzerland)) was mixed with complete Freund's adjuvant (Sigma) in a ratio of 1:1 (v/v) and injected subcutaneously to rabbits. Two boost injections (proteins were mixed 1:1 (v/v) with incomplete Freund's adjuvant) were given two and four weeks later, respectively. Six weeks after the first injection rabbits were bled; the serum was harvested and stored in aliquots at -20 °C. Immunisations were conducted under the registration number 144/2008 in accordance with legal prescriptions.

Western Blotting

Cross-reactivity of rabbit hyperimmune sera produced against MSG1 and porcine α-actin was tested by Western Blot as described previously [13]. Briefly, actin and MSG1 were loaded on 10 % polyacrylamide gels containing 1 % SDS (w/v), and transferred to a nitrocellulose membrane (Whatman Protran®, GE Healthcare). Free binding positions on the membrane were blocked with skim milk (Sigma, 2 % w/v in Tris buffered saline; TBS), and probed by rabbit hyperimmune sera (diluted 1:100 in TBS containing 2 % skim milk powder) produced against recombinant MSG1 and α-actin, respectively. A secondary antibody reactive with pig IgG (Sigma) and coupled to horse radish peroxidase (HRP) diluted 1:5000 in TBS containing 2 % (w/v) skim milk powder was used. The substrate for the HRP was H_2O_2 (Sigma); 4-Chloro-1-naphtol (Sigma) was used as chromogenic agent. The reaction was stopped by adding MQ water (Millipore, Zug, Switzerland).

Search for cross-reactive epitopes

To identify potential cross-reactive epitopes an epitope finder program referred to as NetMHCpan algorithm was used [25]. Published protein sequences of MSG1 (UniProtKB/TrEMBL:Q05G10), porcine α-actin (Swiss-Prot:P68137.1) and porcine β-actin (Swiss-Prot:Q6QAQ1.2) were used as input.

Statistical analysis

Resulting ELISA OD values measured at wavelength λ = 405 nm were compared to each other by linear regression models using the SigmaPlot software, Version 10.0 (SPSS Inc., Chicago, IL, U.S.A). To show independency of values between two groups the unpaired student t-test was applied (significant difference if $P \leq 0.05$).

Authors contributions

KMF performed the experiments with group 1 sera and drafted the manuscript, KH performed the experiments with group 2 sera and helped to draft the manuscript, KHeinritzi did the study design of animal experiments (Group 2) and performed them, the animal experiments of group 1 were designed and organised by MR, LH did the overall study design, planned and coordinated the study, supervised the experiments and helped to draft the manuscript. All authors read and approved the final manuscript.

Acknowledgments

This work was supported both by the Jubiläumsspende (material costs) and the Forschungskredit (PhD salary of KMF) of the University of Zurich, Switzerland.
Anja Hamburger is acknowledged for great technical help.

References

1. Bottiger LE, Westerholm B: **Acquired haemolytic anaemia. I. Incidence and aetiology.** *Acta Med Scand* 1973, **193:**223-226.
2. Sokol RJ, Booker DJ, Stamps R: **The pathology of autoimmune haemolytic anaemia.** *J Clin Pathol* 1992, **45:**1047-1052.
3. Packman CH: **Hemolytic anemia due to warm autoantibodies.** *Blood Rev* 2008, **22:**17-31.
4. Murphy WG, Kelton JG: **Methyldopa-induced autoantibodies against red blood cells.** *Blood Rev* 1988, **2:**36-42.
5. Petz LD: **Cold antibody autoimmune hemolytic anemias.** *Blood Rev* 2008, **22:**1-15.
6. Herold G: **Autoimmunhaemolytische Anämien (AIHA).** In *Innere Medizin.* Edited by Herold G. Köln; 2006: 43-45
7. Hoelzle LE: **Haemotrophic mycoplasmas: recent advances in *Mycoplasma suis*.** *Vet Microbiol* 2008, **130:**215-226.
8. Messick JB: **Hemotrophic mycoplasmas (hemoplasmas): a review and new insights into pathogenic potential.** *Vet Clin Pathol* 2004, **33:**2-13.
9. Yang D, Tai X, Qiu Y, Yun S: **Prevalence of *Eperythrozoon spp*. infection and congenital eperythrozoonosis in humans in Inner Mongolia, China.** *Epidemiol Infect* 2000, **125:**421-426.
10. dos Santos AP, dos Santos RP, Biondo AW, Dora JM, Goldani LZ, de Oliveira ST, de Sa Guimaraes AM, Timenetsky J, de Morais HA, Gonzalez FH, Messick JB: **Hemoplasma infection in HIV-positive patient, Brazil.** *Emerg Infect Dis* 2008, **14:**1922-1924.
11. Hoffmann R, Schmid DO, Hoffmann-Fezer G: **Erythrocyte antibodies in porcine eperythrozoonosis.** *Vet Immunol Immunopathol* 1981, **2:**111-119.
12. Zachary JF, Smith AR: **Experimental porcine eperythrozoonosis: T-lymphocyte suppression and misdirected immune responses.** *Am J Vet Res* 1985, **46:**821-830.
13. Hoelzle LE, Hoelzle K, Ritzmann M, Heinritzi K, Wittenbrink MM: ***Mycoplasma suis* antigens recognized during humoral immune response in experimentally infected pigs.** *Clin Vaccine Immunol* 2006, **13:**116-122.

14. Hoelzle LE, Hoelzle K, Harder A, Ritzmann M, Aupperle H, Schoon HA, Heinritzi K, Wittenbrink MM: **First identification and functional characterization of an immunogenic protein in unculturable haemotrophic Mycoplasmas (*Mycoplasma suis* HspA1).** *FEMS Immunol Med Microbiol* 2007, **49:**215-223.
15. Hoelzle K, Doser S, Ritzmann M, Heinritzi K, Palzer A, Elicker S, Kramer M, Felder KM, Hoelzle LE: **Vaccination with the *Mycoplasma suis* recombinant adhesion protein MSG1 elicits a strong immune response but fails to induce protection in pigs.** *Vaccine* 2009, **27:**5376-5382.
16. Schreiber AD, Frank MM: **Role of antibody and complement in the immune clearance and destruction of erythrocytes. I. In vivo effects of IgG and IgM complement-fixing sites.** *J Clin Invest* 1972, **51:**575-582.
17. Semple JW, Freedman J: **Autoimmune pathogenesis and autoimmune hemolytic anemia.** *Semin Hematol* 2005, **42:**122-130.
18. Genty I, Michel M, Hermine O, Schaeffer A, Godeau B, Rochant H: **[Characteristics of autoimmune hemolytic anemia in adults: retrospective analysis of 83 cases].** *Rev Med Interne* 2002, **23:**901-909.
19. Johnson GD, Holborow EJ, Glynn LE: **Antibody to smooth muscle in patients with liver disease.** *Lancet* 1965, **2:**878-879.
20. Lutz HU: **Homeostatic roles of naturally occurring antibodies: an overview.** *J Autoimmun* 2007, **29:**287-294.
21. Biberfeld G, Gronowicz E: ***Mycoplasma pneumoniae* is a polyclonal B-cell activator.** *Nature* 1976, **261:**238-239.
22. Fossati-Jimack L, Azeredo da Silveira S, Moll T, Kina T, Kuypers FA, Oldenborg PA, Reininger L, Izui S: **Selective increase of autoimmune epitope expression on aged erythrocytes in mice: implications in anti-erythrocyte autoimmune responses.** *J Autoimmun* 2002, **18:**17-25.
23. Groebel K, Hoelzle K, Wittenbrink MM, Ziegler U, Hoelzle LE: ***Mycoplasma suis* invades porcine erythrocytes.** *Infect Immun* 2009, **77:**576-584.
24. Zachary JF, Basgall EJ: **Erythrocyte membrane alterations associated with the attachment and replication of *Eperythrozoon suis:* a light and electron microscopic study.** *Vet Pathol* 1985, **22:**164-170.

25. Hoof I, Peters B, Sidney J, Pedersen LE, Sette A, Lund O, Buus S, Nielsen M: **NetMHCpan, a method for MHC class I binding prediction beyond humans.** *Immunogenetics* 2009, **61:**1-13.
26. Kohm AP, Fuller KG, Miller SD: **Mimicking the way to autoimmunity: an evolving theory of sequence and structural homology.** *Trends Microbiol* 2003, **11:**101-105.
27. Wucherpfennig KW: **Mechanisms for the induction of autoimmunity by infectious agents.** *J Clin Invest* 2001, **108:**1097-1104.
28. Fujinami RS, Oldstone MB: **Amino acid homology between the encephalitogenic site of myelin basic protein and virus: mechanism for autoimmunity.** *Science* 1985, **230:**1043-1045.
29. Fischer JT, Petz LD, Garratty G, Cooper NR: **Correlations between quantitative assay of red cell-bound C3, serologic reactions, and hemolytic anemia.** *Blood* 1974, **44:**359-373.
30. Lucisano Valim YM, Lachmann PJ: **The effect of antibody isotype and antigenic epitope density on the complement-fixing activity of immune complexes: a systematic study using chimaeric anti-NIP antibodies with human Fc regions.** *Clin Exp Immunol* 1991, **84:**1-8.
31. Schenkein HA, Barbour SE, Tew JG: **Cytokines and inflammatory factors regulating immunoglobulin production in aggressive periodontitis.** *Periodontol 2000* 2007, **45:**113-127.
32. Sokol RJ, Hewitt S: **Autoimmune hemolysis: a critical review.** *Crit Rev Oncol Hematol* 1985, **4:**125-154.
33. Hoelzle LE, Adelt D, Hoelzle K, Heinritzi K, Wittenbrink MM: **Development of a diagnostic PCR assay based on novel DNA sequences for the detection of *Mycoplasma suis* (*Eperythrozoon suis*) in porcine blood.** *Vet Microbiol* 2003, **93:**185-196.
34. Hall SM, Cipriano JA, Schoneweis DA, Smith JE, Fenwick BW: **Isolation of infective and non-infective *Eperythrozoon suis* bodies from the whole blood of infected swine.** *Vet Rec* 1988, **123:**651.
35. Hoelzle LE, Hoelzle K, Wittenbrink MM: **Expression of the major outer membrane protein (MOMP) of *Chlamydophila abortus*, *Chlamydophila pecorum*, and *Chlamydia suis* in *Escherichia coli* using an arabinose-**

inducible plasmid vector. *J Vet Med B Infect Dis Vet Public Health* 2003, **50:**383-389.

Figures

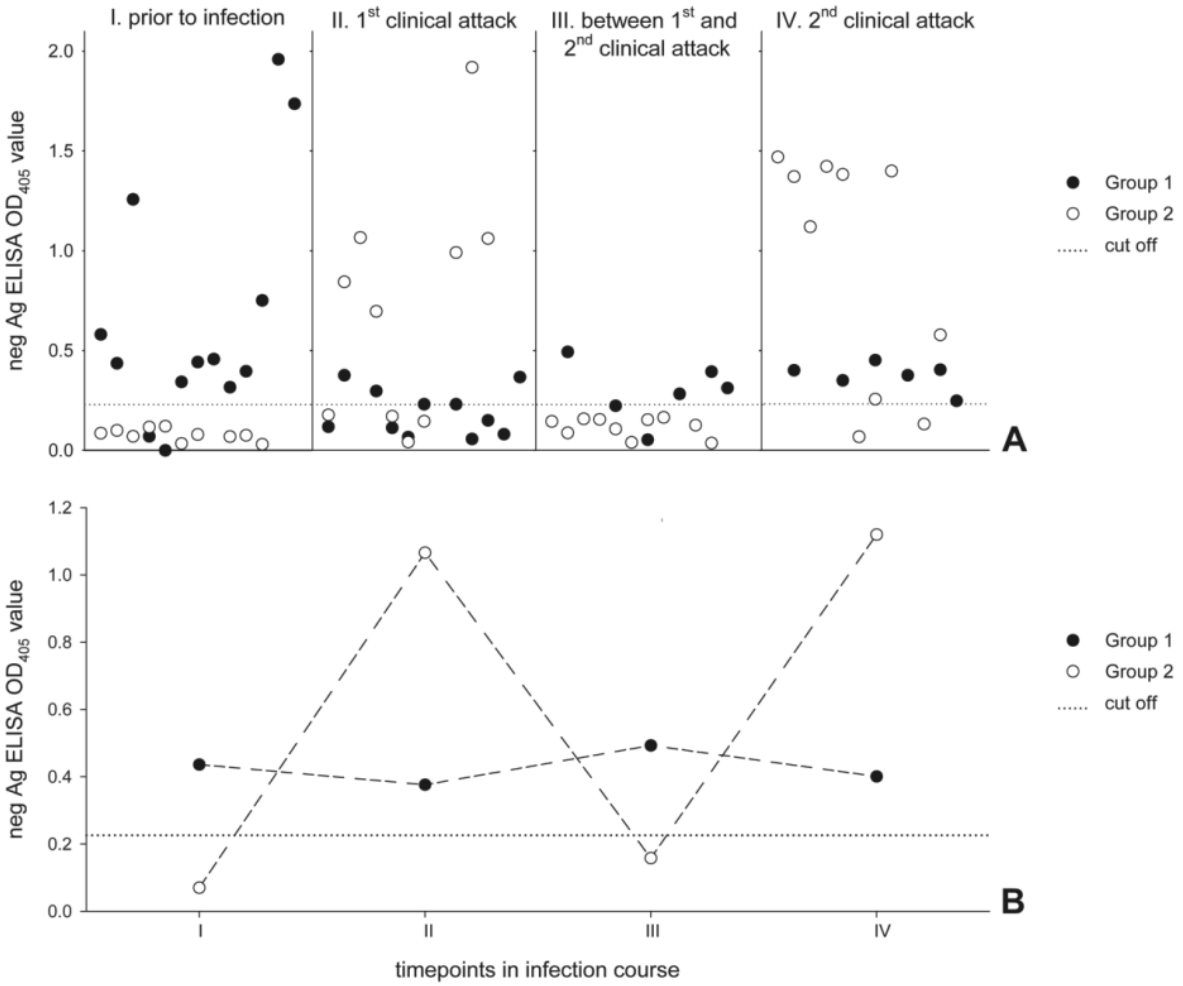

Figure 1

Serum reactivities with neg Ag at several time points (I to IV). A: Group 1 comprises animals that were immunised with MSG1 prior to infection with *M. suis* and splenectomy. Pigs in group 2 were splenectomised and infected with *M. suis*. B: Occurrence of autoreactive antibodies during the time course of infection, representative for one animal of each group.

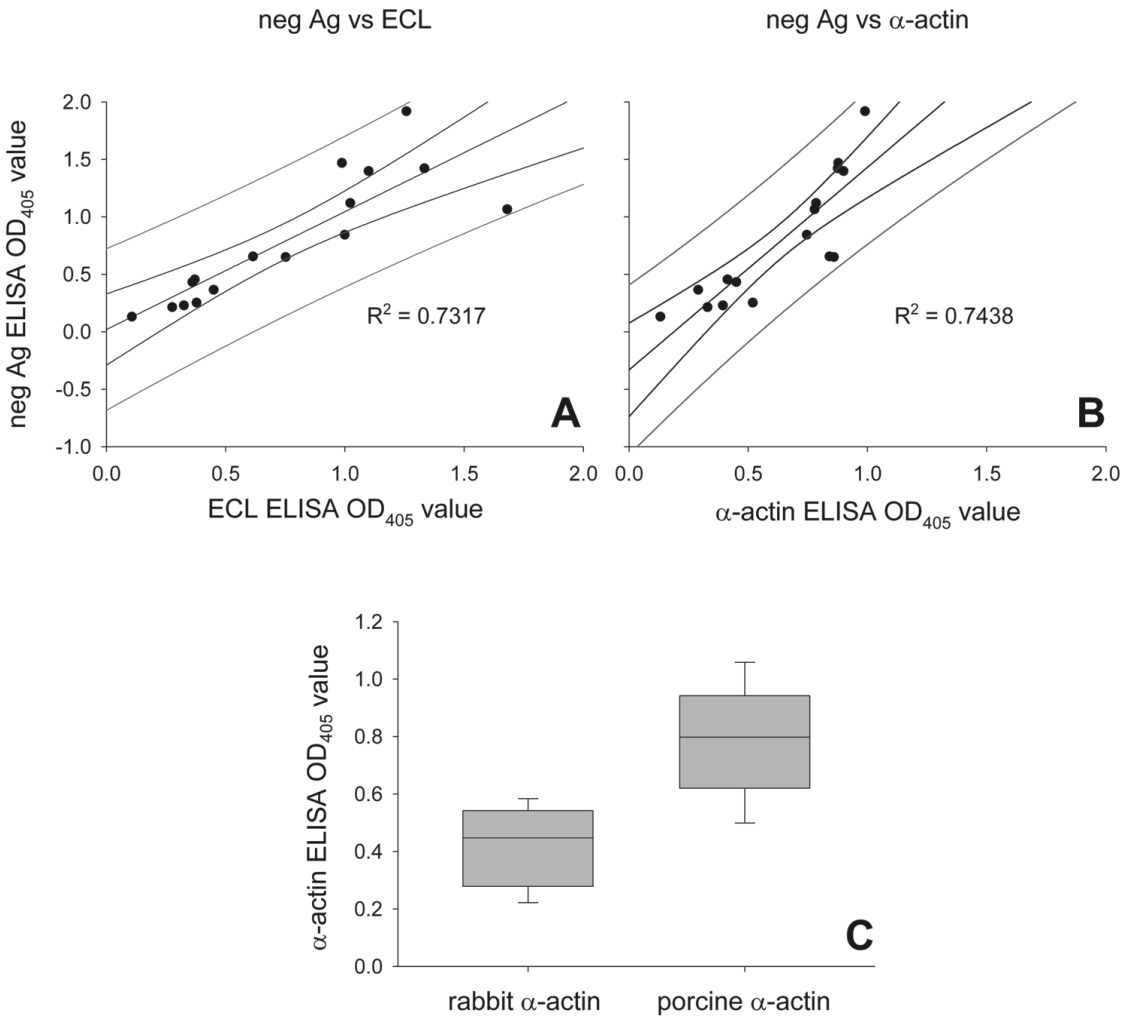

Figure 2

Actin as autoantigen and species specificity. Linear regressions between reactivities with neg Ag and ECL (A) as well as with neg Ag and α-actin (B) are shown for 16 sera. Additionally, 95 % confidence and 95 % prediction bands are shown. C: comparison between reactivities with α-actin of rabbits and pigs.

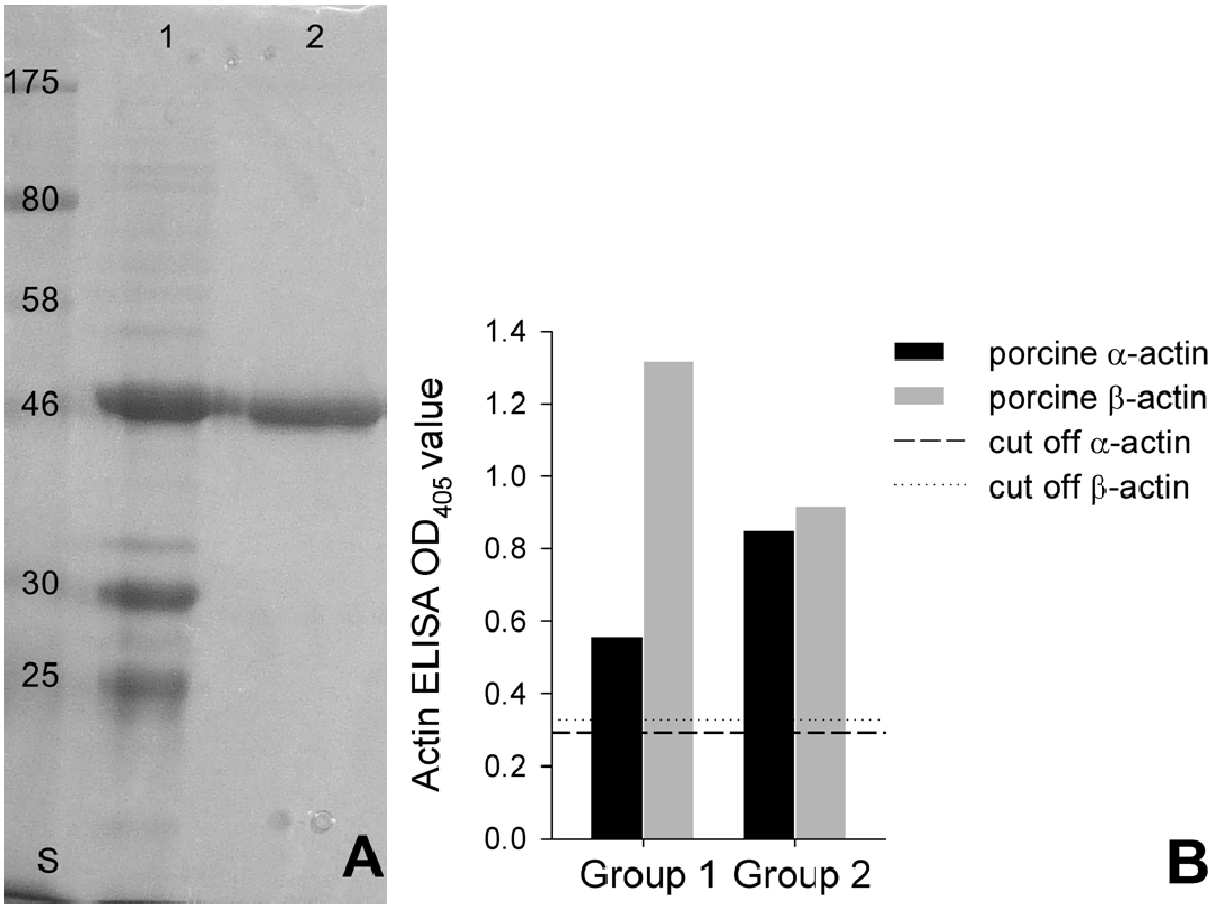

Figure 3

Purification of porcine β-actin and comparison of serum reactivities with α-actin and β-actin. A: Coomassie stained polyacrylamid gel showing purifications of recombinant porcine β-actin. Standard in kDa is given in column S; B: Comparison of reactivity with α-actin and β-actin. Group 1, i.e. immunised animals showed significant stronger reaction with cytoskeletal actin ($p \leq 0.0002$). No difference between α- and β-actin reactivities was observed in sera of group 2 ($p \leq 0.2034$).

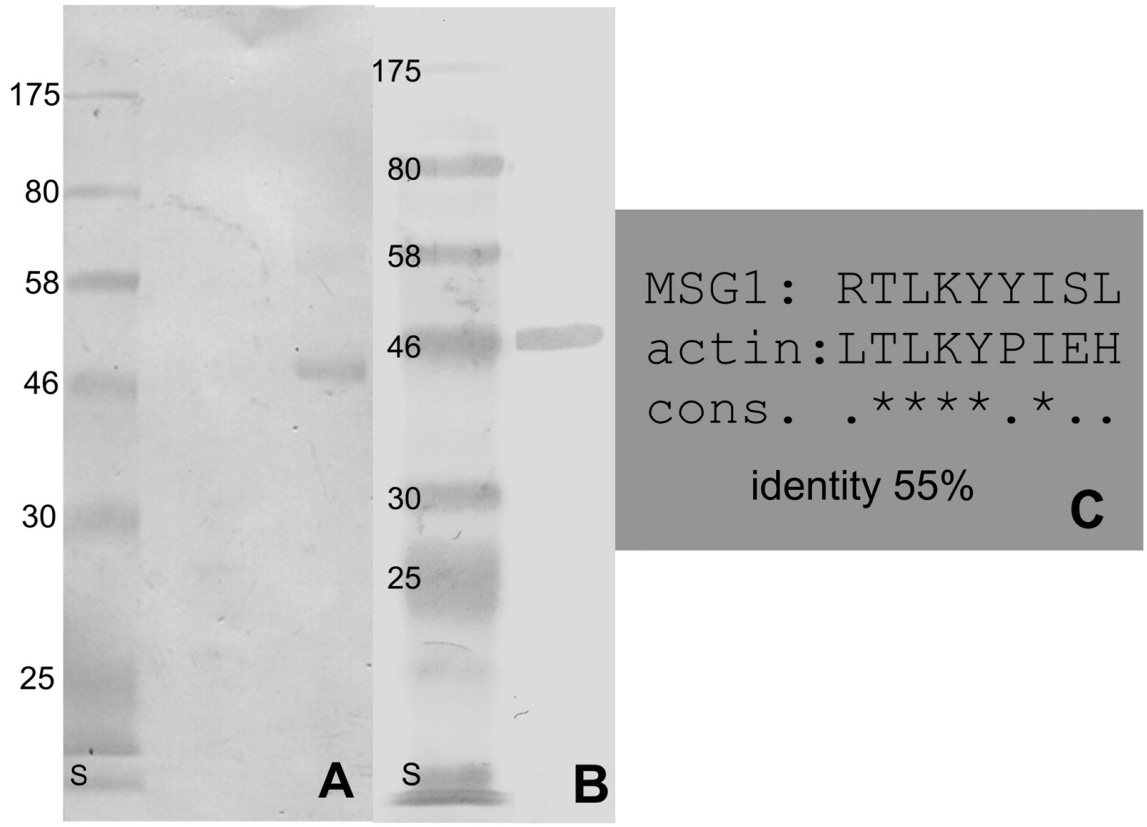

Figure 4

Cross-reactivity of rabbit hyperimmune sera between actin and MSG1. A: Westernblot reactivity of MSG1 with a rabbit serum specific for porcine α-actin; B: Porcine β-actin is detected by a rabbit serum specific for MSG1, column S in A and B indicates protein masses in kDa; C: potential cross-reactive nonapeptides that would be presented by SLA*002 molecule. They share an identity of 55 %.

8.2 Programmed cell death of red blood cells influences development of anaemia in pigs infected with Mycoplasma suis

Kathrin M. Felder, Katharina Hoelzle, Katrin Gröbel, and Ludwig E. Hoelzle

Manuscript in preparation

A reason for shortened life-span of *M. suis* infected red blood cells is eryptosis. The incidence of eryptosis was measured over the infection course of experimental infectious anaemia of pigs and compared to haematological parameters, bacterial load and disease severity.

I collected samples, did the study design, analysed samples for haematological parameters and eryptosis occurrence and wrote the manuscript.

Programmed Cell Death of Red Blood Cells Influences Development of Anaemia in Pigs Infected with *Mycoplasma suis*

Kathrin M. Felder, Katharina Hoelzle, Katrin Groebel, Ludwig E. Hoelzle*

Instiute for Veterinary Bacteriology, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland

Abstract

Severe anaemia is a main clinical sign of pigs infected with *M. suis*. So far, pathogenesis has not been clarified. The involvement of autoreactive mechanisms and direct cell lysis due to *M. suis* was described. Based on literature findings the idea occurred that *M. suis* infection triggered programmed cell death and therefore aggravated anaemia. In erythrocytes, suicidal cell death is referred to as eryptosis and is among other signs characterised by cell shrinkage, microvesiculation and phosphatidyl-serine (PS) exposure on the outer membrane leaflet. In this study, we evaluated the eryptosis occurrence over the time course of infection with *M. suis* and related it with development of anaemia. For this, three *M. suis* strains differing in virulence were used. All three strains were able to induce eryptosis. In pigs infected with the highly virulent strain, anaemia correlated at the beginning with upregulation of eryptosis. The moderately virulent strain seems to be controlled at the beginning by removal of infected cells from the circulation system. Therefore, the progress of anaemia decelerated. Infections with the low virulent strain are controlled by means of eryptosis and anaemia did not occur. Additionally, serum and plasma of an acutely ill pig was shown to induce PS exposure on erythrocytes drawn from healthy pigs.

*Corresponding author: PD Dr. L. E. Hoelzle, Institute for Veterinary Bacteriology, Winterthurerstr. 270, 8057 Zürich. Phone: +41446358613, Fax:+416358912.

INTRODUCTION

The main cause of anaemia in pigs is the infection with *Mycoplasma suis*. The disesase is referred to as infectious anaemia of pigs (IAP). *M. suis* belongs to the group of haemotrophic mycoplasmas that are closely associated with red blood cells of several animals [1, 2]. Clinical signs that are accompanied by high bacterial loads (up to 10^{12} bacteria per ml of blood) are raise of body temperature, hypoglycaemia and severe anaemia. Pigs show laboured breathing due to insufficient oxygen transport and sometimes die of hypoglycaemic shock if untreated. Tetracycline saves pigs from death but *M. suis* is not completely eradicated from its host. The following weeks are characterised by milder anaemia, higher susceptibility to other infections and occurrence of acrocyanosis due to cold agglutinins [3, 4]. The chronic phase is interrupted by *M. suis* growth peaks and the aforementioned clinical signs. These “clinical attacks” get milder at each time and pigs survive without antibiotic therapy from the third attack on [1].

During blood circulation, the integrity of erythrocytes is constantly challenged [5]. They are exposed to oxidative stress in the lung, osmotic changes when travelling through kidney medulla and are squeezed by passing small capillaries. As response, red blood cells can undergo suicidal cell death [6, 7]. This process is referred to as eryptosis and is characterised by cell shrinkage, membrane blebbing, activation of proteases and phosphatidylserine exposure at the outer membrane leaflet [8]. *In vitro*, eryptosis is inhibited by cystein protease inhibitors as Ac-DEVD-CHO or Leuptin [6]. Nevertheless, eryptosis is independent of caspases. Red blood cells indeed were described to contain pro-caspase-3 and pro-caspase-8, but the enzymes are not activated. In erythrocytes, inhibitors of cystein proteases rather target µ-calpain than caspases which indicates that µ-calpain is involved in triggering eryptosis [9].

The anaemia observed in context with *M. suis* infection is caused by at least three mechanisms: (I) direct interaction between *M. suis* and the red blood cell, (II) autoreactive mechanisms and (III) eryptosis [10, 11]. The interaction between the bacterium and its host cell is intimate. The bacteria live and divide on and in erythrocytes [12]. The close contact leads to membrane destruction and structural changes which are signals for macrophage uptake and programmed cell death. The aim of this study was to evaluate eryptosis occurrence in *M. suis* infected pigs and to analyse its impact on development of anaemia, one of the main clinical signs in IAP.

METHODS

Pigs

Weaned piglets (female, “deutsche Landrasse”) were housed in a pen of 20 square meters in groups of four animals. After one week, pigs were splenectomised and allowed to recover. At the age of 7 weeks they were infected by injecting 1 ml of *M. suis* containing blood (10^6 to 10^7 bacterial cells per ml) i.m. Beginning at the day of infection body temperature was recorded daily and pigs were bled for sampling twice a week. Animal experiments were conducted under the registration number 55/2007 with legal prescriptions.

M. suis strains

Three different *M. suis* strains were used for the experiments. They were classified according to their virulence, i.e. low, moderate and high. The low virulent strain is referred to as 146/5 (GenBank:FN391021), is not invasive and the pigs survive if treated with tetracycline. In experimental infections first clinical signs appear between 14 dpi and several weeks dpi. The moderate strain, referred to as 3804 is not invasive but difficult to be controlled with antibiotics. First clinical signs are observed from 10 dpi and pigs survive for several days. The highly virulent strain (GenBank:FN391022) is referred to as KI, invasive and untreatable by means of tetracycline. An infected pig dies within 8 dpi. These animals were exsanguinated on the day of first appearance of high fever. The 16S gene sequence is identical in all three *M. suis* strains.

Quantification of M. suis

Quantification of *M. suis* was performed as described previously [13]. Briefly, DNA was extracted from 200 µl EDTA-anticoagulated blood by the blood and tissue kit (Sigma, Buchs, Switzerland) according to the manufacturer’s recommendations and analysed by a quantitative PCR targeting the *g1* adhesin gene of *M. suis*. In parallel, a standard curve was measured to calculate *M. suis* genome equivalents.

Haematological parameters

White blood cell count including differential blood cell analysis, red blood cell count, haemoglobin concentration and haematocrit values were determined using a 3-DIFF Analyzer (ABX Micros CRP, AxonLab, Baden, Switzerland) according to the

manufacturer's recommendations. EDTA anticoagulated blood was tested within one hour of blood withdrawal.

Quantification of eryptosis

To determine the rate of eryptotic cells the red blood cells were purified from EDTA blood by three washing steps with 1xPBS (400 x g, 5 min, 20 °C). 10^7 cells were stained with PKH-26 (Sigma), a membrane cell linker dye to stain cells with intact cell membranes. To detect cells showing phosphatidyl-serine exposure, the erythrocytes were counterstained with 100 µl AnnexinV-Fluos working solution (Roche) for 15 min. The reaction was stopped by addition of 500 µl HEPES Buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$). Cells were immediately analysed by flow cytometry (FACScalibur, BD Biosciences, Switzerland). 10000 red blood cells were recorded; double stained cells were considered as to be eryptotic and quantified. To perform instrument settings, cells fixed with 4 % Paraformaldehyde (PFA) were used. These cells stain all positive with AnnexinV, a phaenomenon described to occur as well in platelets fixed by means of PFA [14].

In vitro induction of eryptosis

Cells were stained with PKH-26 as described above. 10^6 stained cells were incubated over night at 37 °C with 100 µl of serum or plasma of a *M. suis* infected pig. As controls, serum and plasma obtained from the same pigs before infection with *M. suis* were used. As positive control, cells were incubated with 2.5 mM $CaCl_2$ and 0.5 µM Ionomycin (Sigma) in 1xPBS.

Microscopy

Scanning electron microscopy (SEM) was performed as described previously [12]. Briefly, cells were fixed by means of 1 % Glutaraldehyde immediately after blood withdrawal and stored at 4 °C until use. Cells were settled on carbon coated coverslips, dehydrated by increasing concentrations of acetone and dried. The cells were sputter coated with 20 nm platinum and analysed with a Zeiss Supra 50 VP scanning electron microscope.

Statistical analysis

To test whether two groups differed significantly in eryptosis development; the *P*-values were calculated with the unpaired student t-test. Two groups were considered as significantly different if $P \leq 0.05$.

RESULTS

Instrument settings

One characteristic of eryptotic cells is the breakdown of membrane asymmetry. Specific staining with AnnexinV-Fluos allows the quantification of these cells by FACS. Cells undergoing suicidal cell death differ from necrotic cells by maintaining the plasma membrane integrity. Therefore, a membrane dye staining only cells with intact cell membranes (PKH-26) was used as additional marker. To perform instrument settings on the FACS Calibur eryptosis was induced by incubation with 2.5 mM $CaCl_2$ and 0.5 µM Ionomycin. As controls, cells drawn from healthy pigs were stained.

Visualisation of eryptotic cells

Samples from *M. suis* infected pigs containing eryptotic red blood cells (as seen by a significant higher amount of double stained cells compared to blood of healthy pigs) were analysed by scanning electron microscopy. The characteristic changes as cell shrinkage, change in shape from discocytic to sphaerocytic and microvesiculation were observed (Figure 1A). For quantification, cells were double stained with AnnexinV and PKH-26 and analysed on by flow cytometry. 10000 erythrocytes were taken into consideration (Figure 1B). The amount of double stained cells was recorded (Figure 1C). In all *M. suis* infected animals eryptosis was observed.

Quantification of eryptosis over the time course of an M. suis infection

To evaluate the impact of eryptosis for development of anaemia, pigs were infected with three different strains of *M. suis*, i.e. highly virulent (hv), moderately virulent (mv) and low virulent (lv) and bled twice a week. Haematological parameters were recorded and eryptosis occurrence was tested. Table 1 gives an overview of the haematocrit, bacteraemia and double stained red blood cells of the analysed pigs. In pigs infected with the hv *M. suis* strain the disease progressed extremely fast and symptoms were fatal. In these animals a correlation between severity of anaemia as well as of bacterial load with occurrence of doubled stained cells was observed (Table 1, Figure 2). Short before euthanasia, red blood cells were no more stained with PKH-26. At these time points, the membrane damages were too severe and intravascular lysis occurred. Therefore, the values of eryptotic cells were minimised and no cells were double stained. In blood samples of the pig infected with the mv

M. suis strain eryptosis activity was measurable at day 7 post infectionem. Afterwards, no eryptosis was observed. In parallel, the bacterial load increased and the haematocrit decreased. The animal was treated with tetracycline. At day 16 p.i. the bacterial load was decreased but the anaemia was very severe. The animal had to be euthanised due to poor general state of health. For the animal infected with the lv strain eryptotic activity was measured after three weeks of infection. The bacterial load correlated directly to the percentage of double stained red blood cells. During this time the animal did not show any clinical signs.

In vitro induction of eryptosis

To prove the presence of suicidal cell death mechanisms, red blood cells taken from healthy pigs (n = 3, I to III) were incubated with plasma and serum from acutely ill pigs. The *M. suis* loads of serum and plasma were determined to be 9.74 x 10^8 per ml and 6.08 x 10^{11} per ml, respectively. A significant upregulation of double stained erythrocytes was observed (Table 2). Figure 3 illustrates the amount of double stained cells. The difference between serum and plasma incubations and control incubations was significant ($P \leq 0.05$). Freshly drawn red blood cell did not stain with AnnexinV. In Figure 4 density plots of replica I to III are shown.

DISCUSSION

Interestingly, effector caspases are required for red blood cell formation in erythropoiesis [15]. Daugas et al proposed that the differentiation and enucleation of the red blood cell during development was a sort of abortive apoptosis [9]. He therefore considered the mature erythrocyte as an organelle free mummy created by caspase activation. Until then, researchers believed that mature red blood cells were not able for programmed cell death due to lack of nucleus and mitochondria. It was in 2001 when two independent research groups proved the opposite [6, 7]. Upon stress erythrocytes undergo conformational changes as cell shrinkage, vesiculation and exposure of phosphatidylserine on the outer leaflet of the plasma membrane, all well known features occurring during apoptosis of nucleated cells. The exact mechanisms of eryptosis were then clarified by the group of Lang [5].

Several stimuli and diseases trigger eryptosis [16]. Among these are energy depletion, oxidative stress, osmotic shock, cyclosporine, peptidoglycans, prostaglandin E_2, sepsis, malaria and glucose-6-dehydrogenase-deficiency [17-22].

Upon invasion of red blood cells *Plasmodium falciparum* induces formation of ion channels over the erythrocyte membrane for uptake of nutrients, Na^+ and Ca^{2+} ions and disposal of waste products [17, 23]. This channel formation leads to upregulation of eryptosis, removal of infected cells and therefore removal of bacteria from the body. Suicide therefore represents a defence mechanism against malaria [24]. *M. suis* as well is able for cell invasion [12]. In this study, an increase in red blood cells exhibiting phosphatidylserine on their surface was observed during bacteraemia.

In our study we observed eryptosis as well in pigs infected with the non-invasive *M. suis* strain. Eryptosis in animals infected with this strain therefore seems to be induced by some soluble substances produced by the bacterium. In these pigs anaemia was controlled by eryptosis, not induced. Additional studies are necessary to find such eryptosis-triggering factors of non-invasive *M. suis* cells.

A main clinical sign of acute IAP is severe anaemia caused by several mechanisms. We observed upregulation of autoreactive antibodies targeting actin during disease states with high *M. suis* loads [11]. These antibodies can bind actin only if the cell is damaged and its actin gets accessible. The close association of *M. suis* cells with the erythrocyte leads to so far unknown changes in the cytoskeleton and the cell membrane. A possible explanation would be that channel formation by the invader

could induce such changes. Therefore, a combination of increased accessibility of actin and eryptosis rate is likely to account for diminution of erythrocytes and therefore severe anaemia.

The detected patterns of eryptosis occurrence differed in pigs infected with different *M. suis* strains. In infections with a highly virulent strain, the eryptosis process is upregulated but fails to control the infection. Erythrocytes are lysed intravascularly by some other mechanism as autoreactive antibodies targeting the actin or direct lysis by *M. suis* [11]. In the pig infected with the moderate *M. suis* strain eryptosis was observed at the beginning (7 dpi). In blood samples taken later no significant amount of double stained cells was observed. However, the serum taken at day 14 pi, when acute clinical signs were observed, was able to induce eryptosis in erythrocytes from healthy, *M. suis* negative pigs. The pig infected with the low virulent *M. suis* strain never showed clinical signs. Eryptosis occurrence was stated from day 21 to day 35 pi. During this time a measurable load of *M. suis* cells was detected in the blood. Here, eryptosis was successful in defence against the infection. The haematocrit slightly decreased. Pigs in this study used eryptosis as the first line of defence against *M. suis* cells invading red blood cells. Later, when *M. suis* was able to growth the mechanism failed and infection got fatal. Therefore we state, that early anaemia is due to sacrifice of infected red blood cells in favour of the individual. Potential removal of red blood cells by phagocytosis in early anaemia was already discussed in 1985 by Zachary and Smith but no experimental proof was given [3]. In this study we confirm the influence of programmed cell death and therefore phagocytosis. Later in the experimental infection, severity of anaemia directly correlated with *M. suis* load. Other mechanisms i.e. a combination of direct lysis and misled immune response against host actin took over.

Eryptosis was inducible in erythrocytes from healthy animals by overnight incubation with serum and plasma samples taken from acutely ill pigs. Therefore, the *Mycoplasma* cells or a stress signal present in the serum and plasma, respectively, is responsible for induction of the death machinery. Noteworthy, a higher rate of eryptosis occurrence in human sepsis patients was reported [19]. Authors described that erythrocytes of healthy volunteers incubated with plasma from sepsis patients triggered phosphatidylserine exposure and therefore eryptosis. The effect was attributed to accumulation of sphingomyelinase-induced intraerythrocytic ceramide [25]. The reason for sepsis in these patients was diverse. In 8 patients a bacterial

causative agent was identified, in one case it was *M. pneumoniae*. However, the plasma samples used were not treated to remove bacteria.

In context with *M. suis* infections it is not clear what kind of signal is used for triggering suicidal red blood cell death. In both samples, *M. suis* cells were present. Further experiments have to be performed with *M. suis* free serum and plasma samples. A problem is the sterilisation of plasma and serum samples. Filtration is not the method of choice out of two reasons. First, mycoplasmas pass filters with 0.2 µm pore size, and second, serum and plasma samples are highly viscous. Therefore, it is impossible to use filters with smaller pore sizes. Generally, sera are inactivated by means of addition of 0.05 % of sodium azide (NaN_3). However, no significant differentiation considering double staining of red blood cells was observed compared to the controls if sera and plasma samples were treated by means of NaN_3 (data not shown). With this inactivation method bacteria are killed but not removed from the samples. Therefore, eryptosis triggering factors still could be present and be active. A further possibility is heat inactivation. Here, the unintentional deactivation of potential eryptosis triggering factors has to be taken into account.

Normally, senescent red blood cells are recognised by macrophages and removed from circulation in the spleen. Our infection model is the splenectomised pig. Theoretically, an accumulation of senescent cells due to the lack of the spleen is thinkable. Therefore, we included a splenectomised, non infected pig in our study and additionally analysed several samples from splenectomised pigs prior to infection with *M. suis*. There was no increase in red blood cells exhibiting phosphatidylserine on their surface compared to healthy, non splenectomised pigs.

In this study we observed the occurrence of eryptosis in response to infection with *M. suis.* Independent on the virulence of the *M. suis* strains used eryptosis was observed. However, the amount of eryptotic cells and the time-point of occurrence differed in animals infected with different strains. Due to correlation of severity of anemia with the occurrence of eryptotic cells in mv and hv strains we state that the early anaemia is due to suicidal destruction of red blood cells. Later on, other mechanisms as autoreactive antibodies and direct lysis due to cell membrane damage take over. In pigs infected with the lv *M. suis* strain eryptosis occurred, but anaemia was not observed. Here, eryptosis seems to be an effective defence mechanism against *M. suis* infection. Eryptosis was inducible in healthy erythrocytes by incubation with serum and plasma of an infected animal. It remains to be

elucidated whether the bacteria directly or some soluble substances present in the plasma are the triggering factor for upregulation of eryptosis in *M. suis* infected pigs.

ACKNOWLEDGMENTS

The study was supported by the Forschungskredit of the University of Zurich, Zurich.

REFERENCES

1. LE Hoelzle: **Haemotrophic mycoplasmas: recent advances in *Mycoplasma suis***. *Vet Microbiol* 2008, **130**:215-26.
2. JB Messick: **Hemotrophic mycoplasmas (hemoplasmas): a review and new insights into pathogenic potential**. *Vet Clin Pathol* 2004, **33**:2-13.
3. JF Zachary, AR Smith: **Experimental porcine eperythrozoonosis: T-lymphocyte suppression and misdirected immune responses**. *Am J Vet Res* 1985, **46**:821-30.
4. P Schmidt, B Kaspers, A Jungling, K Heinritzi, U Losch: **Isolation of cold agglutinins in *Eperythrozoon suis*-infected pigs**. *Vet Immunol Immunopathol* 1992, **31**:195-201.
5. KS Lang, PA Lang, C Bauer, C Duranton, T Wieder, SM Huber, F Lang: **Mechanisms of suicidal erythrocyte death**. *Cell Physiol Biochem* 2005, **15**:195-202.
6. D Bratosin, J Estaquier, F Petit, D Arnoult, B Quatannens, JP Tissier, C Slomianny, C Sartiaux, C Alonso, JJ Huart, et al: **Programmed cell death in mature erythrocytes: a model for investigating death effector pathways operating in the absence of mitochondria**. *Cell Death Differ* 2001, **8**:1143-56.
7. CP Berg, IH Engels, A Rothbart, K Lauber, A Renz, SF Schlosser, K Schulze-Osthoff, S Wesselborg: **Human mature red blood cells express caspase-3 and caspase-8, but are devoid of mitochondrial regulators of apoptosis**. *Cell Death Differ* 2001, **8**:1197-206.
8. F Lang, KS Lang, PA Lang, SM Huber, T Wieder: **Mechanisms and significance of eryptosis**. *Antioxid Redox Signal* 2006, **8**:1183-92.
9. E Daugas, C Cande, G Kroemer: **Erythrocytes: death of a mummy**. *Cell Death Differ* 2001, **8**:1131-3.
10. LE Hoelzle, K Hoelzle, M Helbling, H Aupperle, HA Schoon, M Ritzmann, K Heinritzi, KM Felder, MM Wittenbrink: **MSG1, a surface-localised protein of *Mycoplasma suis* is involved in the adhesion to erythrocytes**. *Microbes Infect* 2007, **9**:466-74.
11. KM Felder, Hoelzle, K., Heinritzi, K., Ritzmann, M., Hoelzle, L.E.: **Antibodies to actin in pigs suffering from autoimmune haemolytic anaemia**. *BMC Vet Res* 2010, **submitted**.

12. K Groebel, K Hoelzle, MM Wittenbrink, U Ziegler, LE Hoelzle: ***Mycoplasma suis* invades porcine erythrocytes**. *Infect Immun* 2009, **77**:576-84.
13. LE Hoelzle, M Helbling, K Hoelzle, M Ritzmann, K Heinritzi, MM Wittenbrink: **First LightCycler real-time PCR assay for the quantitative detection of *Mycoplasma suis* in clinical samples**. *J Microbiol Methods* 2007, **70**:346-54.
14. K Wong, X Li, Y Ma: **Paraformaldehyde induces elevation of intracellular calcium and phosphatidylserine externalization in platelets**. *Thromb Res* 2006, **117**:537-42.
15. Y Zermati, C Garrido, S Amsellem, S Fishelson, D Bouscary, F Valensi, B Varet, E Solary, O Hermine: **Caspase activation is required for terminal erythroid differentiation**. *J Exp Med* 2001, **193**:247-54.
16. F Lang, E Gulbins, H Lerche, SM Huber, DS Kempe, M Foller: **Eryptosis, a window to systemic disease**. *Cell Physiol Biochem* 2008, **22**:373-80.
17. VB Brand, CD Sandu, C Duranton, V Tanneur, KS Lang, SM Huber, F Lang: **Dependence of *Plasmodium falciparum* in vitro growth on the cation permeability of the human host erythrocyte**. *Cell Physiol Biochem* 2003, **13**:347-56.
18. M Foller, R Biswas, H Mahmud, A Akel, E Shumilina, T Wieder, F Goetz, F Lang: **Effect of peptidoglycans on erythrocyte survival**. *Int J Med Microbiol* 2009, **299**:75-85.
19. DS Kempe, A Akel, PA Lang, T Hermle, R Biswas, J Muresanu, B Friedrich, P Dreischer, C Wolz, U Schumacher, et al: **Suicidal erythrocyte death in sepsis**. *J Mol Med* 2007, **85**:273-81.
20. KS Lang, C Duranton, H Poehlmann, S Myssina, C Bauer, F Lang, T Wieder, SM Huber: **Cation channels trigger apoptotic death of erythrocytes**. *Cell Death Differ* 2003, **10**:249-56.
21. KS Lang, B Roll, S Myssina, M Schittenhelm, HG Scheel-Walter, L Kanz, J Fritz, F Lang, SM Huber, T Wieder: **Enhanced erythrocyte apoptosis in sickle cell anemia, thalassemia and glucose-6-phosphate dehydrogenase deficiency**. *Cell Physiol Biochem* 2002, **12**:365-72.
22. PA Lang, DS Kempe, S Myssina, V Tanneur, C Birka, S Laufer, F Lang, T Wieder, SM Huber: **PGE(2) in the regulation of programmed erythrocyte death**. *Cell Death Differ* 2005, **12**:415-28.

23. K Kirk: **Membrane transport in the malaria-infected erythrocyte**. *Physiol Rev* 2001, **81**:495-537.

24. M Foller, D Bobbala, S Koka, SM Huber, E Gulbins, F Lang: **Suicide for survival-death of infected erythrocytes as a host mechanism to survive malaria**. *Cell Physiol Biochem* 2009, **24**:133-40.

25. KS Lang, S Myssina, V Brand, C Sandu, PA Lang, S Berchtold, SM Huber, F Lang, T Wieder: **Involvement of ceramide in hyperosmotic shock-induced death of erythrocytes**. *Cell Death Differ* 2004, **11**:231-43.

FIGURES AND TABLES

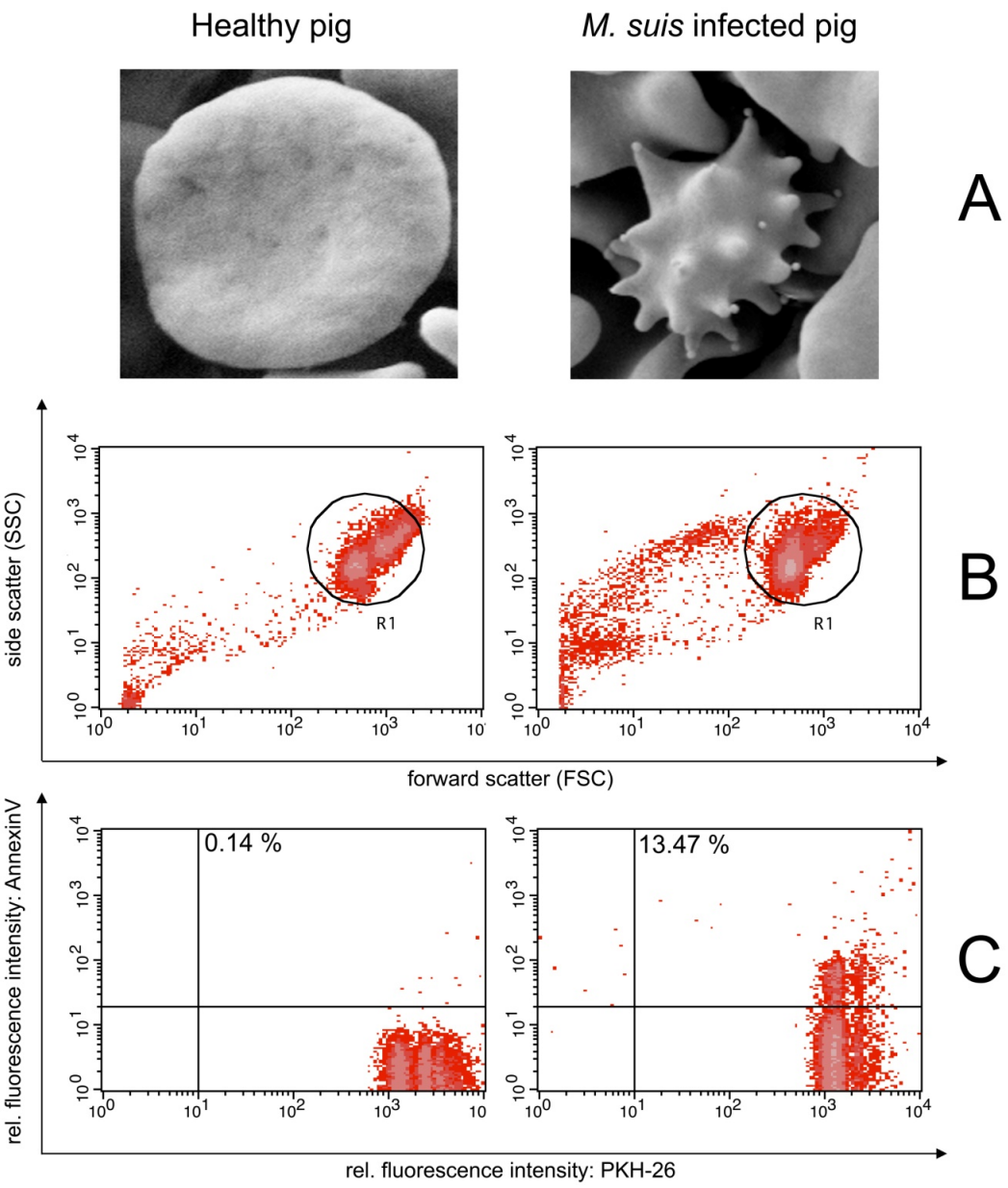

Figure 1 Quantification of eryptotic cells infected with the highly virulent *M. suis* strain. A: Scanning electron micrographs of a discoid, normal red blood cell and a sphaeric eryptotic cell showing microvesiculation are shown. B: Density plots of the erythrocyte population. 10000 cells in gate R1 were considered for quantifications. C: Density plots showing the relative fluorescence intensities of cells in gate R1. The eryptotic and therefore double stained cells are found in the upper right quadrant. Numbers indicate percentage of double stained cells in gate R1.

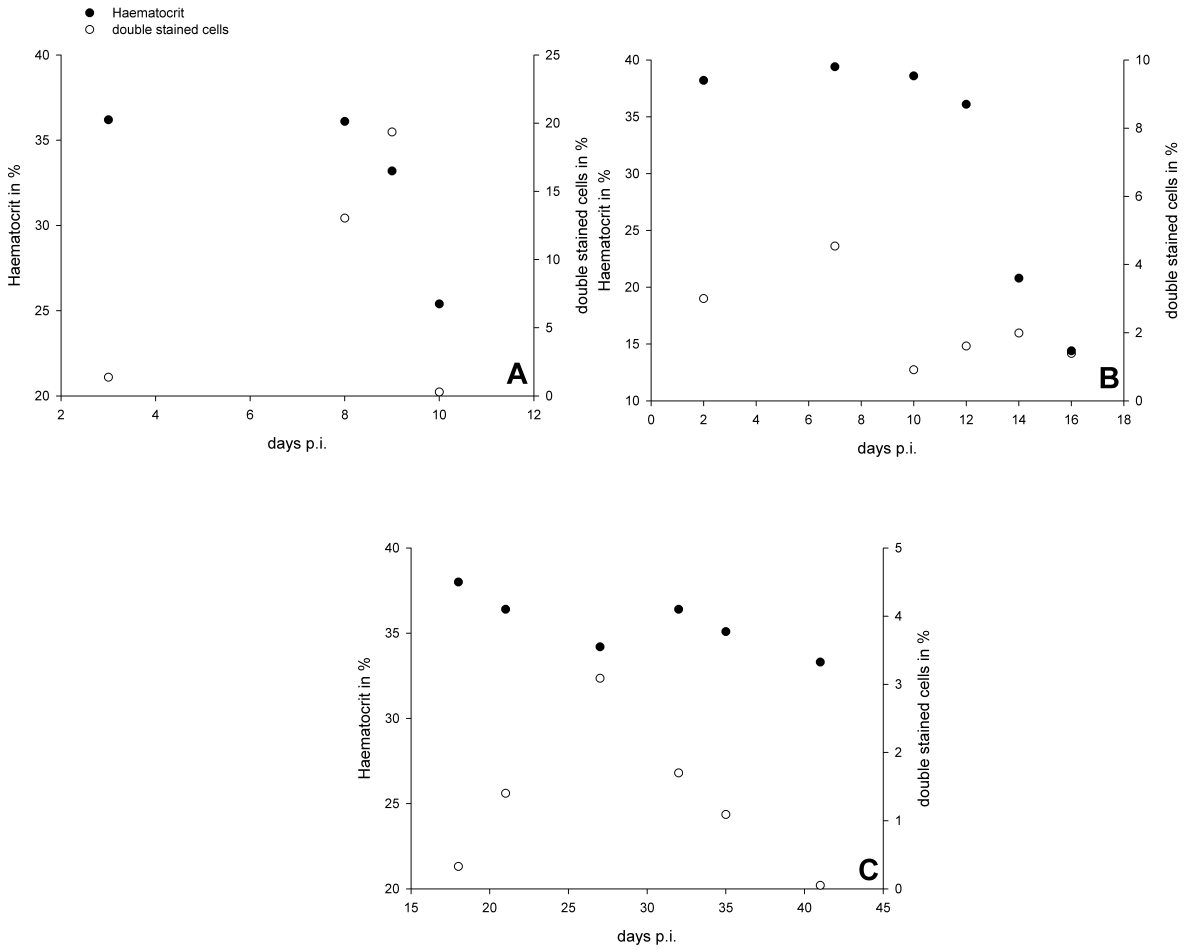

Figure 2 Correlation of doubled stained red blood cells with haematocrits. A: Infection with the highly virulent strain. The severity of anaemia negatively correlates with the amount of double stained red blood cells. At day 10 p.i. the cells did no more stain with PKH-26 due to severe damage and intravascular lysis. B: Infection with the moderately virulent *M. suis* strain. At the beginning of the infection course eryptosis is upregulated. Later, anaemia progresses without appreciable eryptotic activity. C: Infection with the low virulent *M. suis* strain. Eryptosis is observed from day 21 post infection. Haematocrit levels are constant.

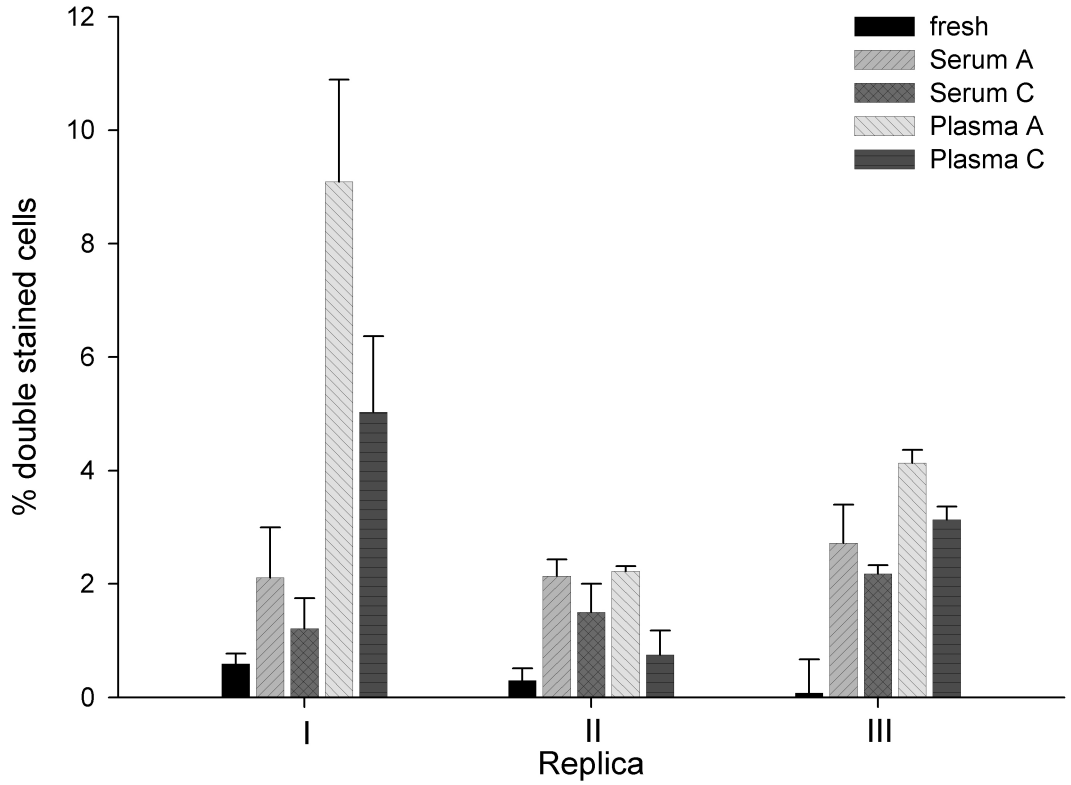

Figure 3 Incubations of red blood cells with serum and plasma from acutely ill and healthy pigs. Bars show means of percentage of double stained red blood cells. Additionally, the error bars are shown. Fresh: cells were stained immediately after blood withdrawal. Serum and Plasma A: cells were incubated over night with serum and plasma samples obtained from an acutely ill pig. Serum and Plasma C: As controls, cells were incubated with samples obtained from the same pig but prior to *M. suis* infection. The differences between A and C were significant as indicated by *P*-values < 0.05 (Table 2).

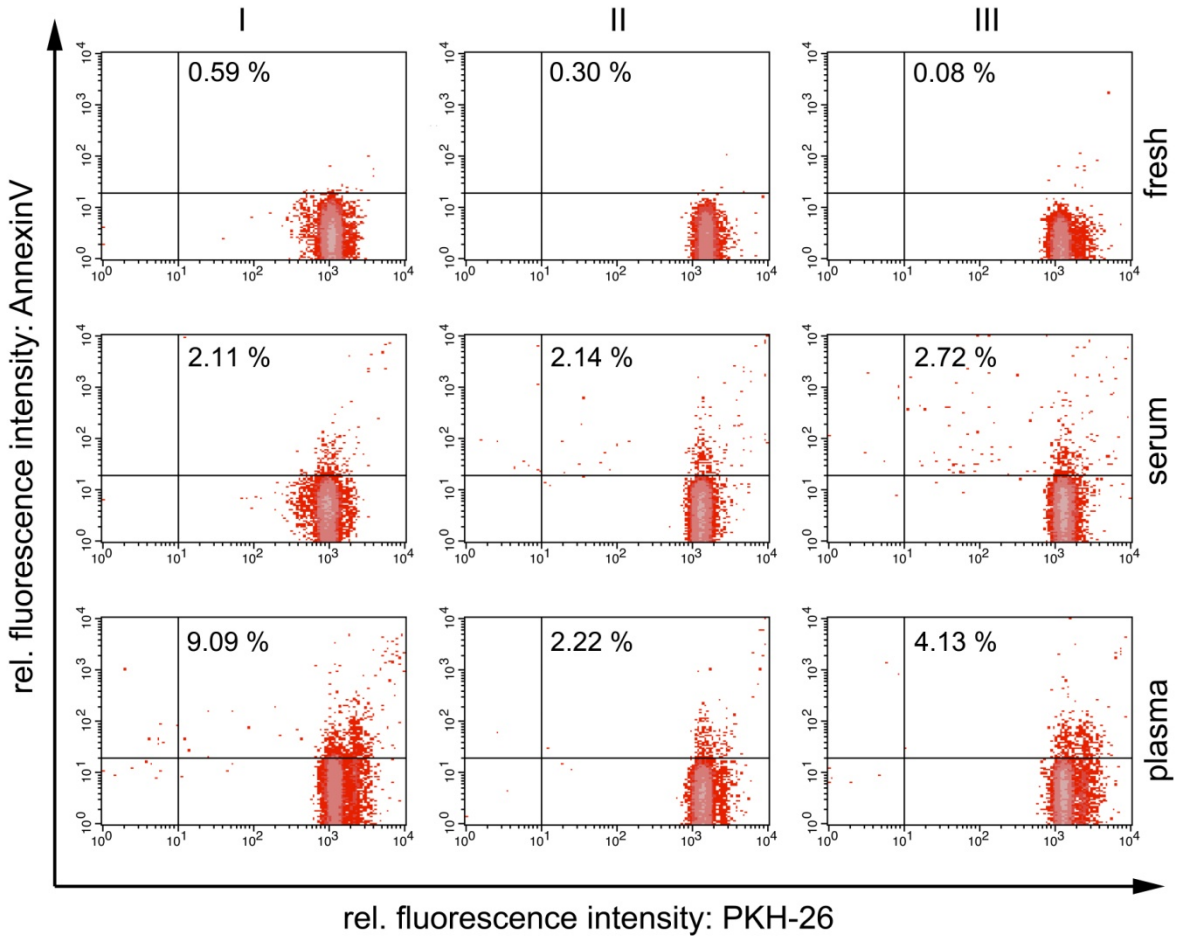

Figure 4 Density plots of double stained red blood cells that were stained with AnnexinV and PKH-26 immediately after withdrawal or after incubation with serum or plasma from an acutely ill pig. Cells appearing in the upper right quadrant of each panel were considered as eryptotic due to double staining. Numbers indicate percentage of 10000 red blood cells.

Table 1

Overview of data obtained. Pigs were infected with three different *M. suis* strains. Eryptosis activity was compared to severity of anaemia (haematocrit) and bacterial load.

Animal	dpi[1]	% dsc[2]	Bacterial load / ml blood	Haematocrit	*M. suis* strain	status
1828	3	0.29	5.32E+03	36.3	hv[3]	
1828	8	18.61	1.61E+11	25.5	hv	euthanised
1846	3	1.37	2.42E+03	36.2	hv	
1846	8	13.04	2.38E+09	36.1	hv	
1846	9	19.35	2.03E+10	33.2	hv	
1846	10	0.29	3.62E+10	25.4	hv	euthanised
5271	2	0.3	4.83E+02	38.2	mv[4]	
5271	7	4.54	1.10E+03	39.4	mv	
5271	10	0.91	7.66E+05	38.6	mv	
5271	12	1.61	1.40E+11	36.1	mv	
5271	14	1.99	7.22E+11	20.8	mv	
5271	16	1.39	6.10E+07	14.4	mv	euthanised
5275	18	0.33	0	38.0	lv[5]	
5275	21	1.40	0	36.4	lv	
5275	27	3.09	6.25E+03	34.2	lv	
5275	32	1.70	1.06E+03	36.4	lv	
5275	35	1.09	2.60E+01	35.1	lv	
5275	41	0.05	0	33.3	lv	survived
5280	2	0.15	2.47E+05	35.2	hv	
5280	7	6.55	7.65E+03	36.7	hv	
5280	12	15.6	8.42E+08	28.6	hv	euthanised

[1] days post infectionem, [2] percent of double stained red blood cells, [3] highly virulent, [4] moderately virulent, [5] low virulent.

Table 2

Double stained red blood cells after incubation with serum or plasma, respectively. 10^6 purified red blood cells from healthy pigs were incubated over night with 100 µl of serum or plasma from an acutely ill pig (serum A, plasma A). Accordingly, cells were incubated with control preparations from the same pig taken prior to *M. suis* infection (serum C, plasma C). *P*-values indicate significance between experimental and control incubations for each sample if ≤ 0.05. Staining properties of cells was evaluated as well before incubation (fresh).

	I		II		III	
	% dsc	*P* value	% dsc	*P* value	% dsc	*P* value
fresh	0.59 ± 0.18		0.30 ± 0.21		0.08 ± 0.04	
serum A	2.11 ± 0.89	0.0003	2.11 ± 0.29	0.0085	2.72 ± 0.68	0.003
serum C	1.21 ± 0.54		1.50 ± 0.50		2.18 ± 0.15	
plasma A	9.09 ± 1.80	0.0002	2.22 ± 0.09	0.0014	4.13 ± 0.23	0.0005
plasma C	5.03 ± 1.34		0.75 ± 0.43		3.13 ± 0.23	

I – III Replica, dsc: double stained red blood cells.

8.3 MSG1, a surface-localised protein of Mycoplasma suis is involved in the adhesion to erythrocytes

Microb Infect. 2007; 9:466-474.

Ludwig E. Hoelzle, Katharina Hoelzle, Marianne Helbling, Heike Aupperle, Heinz A. Schoon, Mathias Ritzmann, Karl Heinritzi, Kathrin M. Felder, Max M. Wittenbrink.

This publication describes the identification and characterization of MSG1 as a *M. suis* protein involved in attachment to the erythrocyte. The protein is analogous to GAPDH and is surface-localised in *M. suis* as well as in *E. coli* transformants. *E. coli* transformants expressing a fusion protein of MSG1 and GFP were used for functional studies concerning the adhesion process to red blood cells.

I performed the FACS analysis to evaluate adhesion of recombinant MSG1_GFP expressing *E. coli* cells to red blood cells.

ELSEVIER

INSTITUT PASTEUR

Microbes and Infection 9 (2007) 466–474

www.elsevier.com/locate/micinf

Original article

MSG1, a surface-localised protein of *Mycoplasma suis* is involved in the adhesion to erythrocytes

Ludwig E. Hoelzle [a,*], Katharina Hoelzle [a], Marianne Helbling [a], Heike Aupperle [b], Heinz A. Schoon [b], Mathias Ritzmann [c], Karl Heinritzi [c], Kathrin M. Felder [a], Max M. Wittenbrink [a]

[a] *Institute of Veterinary Bacteriology, Vetsuisse Faculty, University of Zurich, Winterthurerstrasse 270, 8057 Zurich, Switzerland*
[b] *Institute of Pathology, Faculty of Veterinary Medicine, University of Leipzig, Leipzig, Germany*
[c] *Clinic for Swine, Ludwig-Maximilians-University Munich, Munich, Germany*

Received 25 October 2006; accepted 10 January 2007
Available online 20 January 2007

Abstract

Mycoplasma suis is a member of the group of uncultivable haemoplasmas which colonise erythrocytes of a wide range of vertebrates. Adhesion to erythrocytes is the crucial step in the unique haemoplasma life cycle. Due to the lack of a cultivation system, no adhesion structures have been identified so far. In order to determine potential adhesion molecules of *M. suis*, we screened genomic *M. suis* libraries. The protein MSG1 with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) similarity was identified. The encoding gene *msg*1 is 1011 bp in size. The overall homology of the deduced amino acid sequence to GAPDHs of other pathogenic mycoplasmas ranged from 52.6% to 54.5%. Recombinant MSG1 expressed in *Escherichia coli* exhibited GAPDH activity. Immunoblot and immunoelectron microscopy analyses using antibodies against rMSG1 verified the membrane and surface localisation of native MSG1 in *M. suis*. Furthermore, we showed that rMSG1 binds to erythrocyte lysate in a dose-dependent manner. *E. coli* transformants which express MSG1 on their surface acquire the ability to adhere to porcine erythrocytes. This adhesion could be specifically and significantly inhibited by rMSG1 and antibodies to MSG1. In conclusion, our studies indicate that the membrane-associated MSG1 represents the first putative adhesion protein identified in the group of haemoplasmas.

Keywords: Haemoplasma; *Mycoplasma suis*; MSG1; Adhesion to erythrocytes; GAPDH activity

1. Introduction

Mycoplasma (M.) suis belongs to the hitherto uncultivable haemotrophic mycoplasmas (haemoplasma) that parasitise the red blood cells (RBCs) of a wide range of vertebrates [1,2]. Pathogenic mycoplasmas are primarily surface parasites on the epithelial cells of the respiratory and urogenital tracts of humans and animals. In contrast, the life cycle of haemoplasmas on the surface of RBCs is unique and therefore, it is evident that these haemotrophic pathogens must have unique features allowing them to colonise and replicate on the RBCs. It is well known from electron microscopic studies that *M. suis* and porcine RBCs are in close contact. This interaction is accompanied by severe deformations of the RBC surface in the form of prominent pits, trenches, and invaginations [3–5]. Within these deformed membrane areas *M. suis* organisms are intimately associated with, but distinctly separated from the RBC membrane by a 30-nm electron lucent zone. In this zone, fine fibrillar attachments between both partners could be observed [1]. However, the mechanisms of adhesion and replication of *M. suis* on RBCs as well as the nature and pathogenic significance of metabolic interchanges between the agent and the target cells are completely unknown to date. In general, mycoplasmas employ various attachment structures to

* Corresponding author. Tel.: +41 44 635 8613; fax: +41 44 635 8912.
E-mail address: lhoelzle@vetbakt.unizh.ch (L.E. Hoelzle).

1286-4579/$ - see front matter © 2007 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.micinf.2007.01.004

initiate contact with host cells and several adhesion and cytadherence-associated proteins have been identified. For example, *Mycoplasma pneumoniae* and *Mycoplasma gallisepticum* utilise special tip organelles composed of a network of proteins which mediate adherence to the receptors on target cells [6–8]. Electron microscopic studies have shown that haemoplasmas obviously lack these distinct tip structures [1]. Therefore, alternative mechanisms must be responsible for the surface parasitism of haemoplasmas. The identification and characterisation of *M. suis* adhesins will improve our understanding of the crucial pathogenic mechanisms utilised by the organism and is essential at the onset of studies on the host–pathogen interactions of haemoplasmas. Furthermore, these insights will provide crucial data for the development of vaccines for controlling *M. suis*-induced diseases. Currently, however, the detection of *M. suis* proteins that may mediate the interaction of the bacterium with the host erythrocytes is hampered by the lack of an in vitro cultivation system for *M. suis* providing pure bacterial cells.

In the present study, genomic *M. suis* libraries were screened in order to identify putative adhesion proteins. One clone was found to contain an *M. suis* gene demonstrating homology with the glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) of *Mycoplasma penetrans*. In numerous pathogens GAPDH-like proteins are known to fulfill multiple functions in different subcellular locations, i.e. mucin binding in *Mycoplasma genitalium* as a surface-localised protein [9] or myosin and actin binding in group A streptococci (membrane-bound GAPDH) [10]. Thus, the corresponding protein from *M. suis*, designated as MSG1, was chosen for further characterisation. Furthermore, MSG1 with putative GAPDH functions attracted our special attention due to its glycolytic activity. During the acute phase of *M. suis* infections, studies of the blood parameters showed a decrease of glucose in the blood of infected pigs. This life-threatening hypoglycaemia can be observed in naturally infected pigs as well as in experimentally infected pigs [5,11,12]. When the blood glucose level drops to less than 10 mg/dl, pigs commonly suffer from convulsions and coma followed by death. Decreased blood glucose concentrations are clearly associated with the metabolism of *M. suis* [12,13]. Nevertheless, *M. suis* proteins with a glycolytic activity have not been identified to date.

Here, we describe the identification and characterisation of an *M. suis* adhesion factor called MSG1 (*M. suis* GAPDH-like protein 1) with functional properties similar to bacterial GAPDHs. This is the first report on a virulence factor within the group of haemoplasmas.

2. Materials and methods

2.1. Bacterial strains, plasmids and experimental pig sera

M. suis cells were obtained from experimentally infected pigs as previously described [14,15]. *Escherichia coli* K12 strains were Top10 and LMG194 (Invitrogen, Basel, Switzerland). The plasmids pUC19 (Roche-Diagnostics, Rotkreuz, Switzerland), pGFP (Clontech, Allschwil, Switzerland) and pBad*Myc*His (Invitrogen) were used. Experimental sera were available from previous studies [15].

2.2. Library construction

DNA library construction was performed by Medigenomix (Martinsried, Germany). *M. suis* DNA fragments averaging from 1.5 kb to 3.0 kb were ligated into the pUC19 vector. Two hundred clones were randomly chosen for sequencing. Sequences were analysed using BLASTn/BLASTx, FASTA algorithms (Biocomputing, University Zurich, www-bio.unizh.ch/), and an ORF finder program (www.ncbi.nlm.nih.gov/projects/gorf/).

2.3. Cloning of msg1 and gfp

To circumvent the *Mycoplasma* specific translational barrier of the UGA codon, the *msg*1 sequence was adapted to the codon usage of *E. coli* and de novo synthesised (Medigenomix). The de novo *msg*1 was ligated into the pBad*Myc*His vector and transformed into *E. coli* LMG194 (*E. coli_msg*1). To construct an MSG1 fusion protein with GFP we amplified the *gfp* open reading frame from the plasmid pGFP. The amplified *gfp* was ligated into the plasmid pBad_*msg*1 and pBad and transformed into *E. coli* LMG194. The *E. coli* transformants were named *E. coli_msg*1*_gfp* and *E. coli_gfp*, respectively.

2.4. Expression and purification of recombinant MSG1

Expression was induced by the addition of 0.02% arabinose. *E. coli* cultures were further incubated for 2 h at 37 °C. His-tagged proteins were purified by nickel affinity chromatography (Qiagen, Hombrechtikon, Switzerland) from the cytoplasmic and outer membrane compartments as previously described [16]. Expression of GFP was analysed by fluorescent microscopy (Olympus fluorescence microscope BX40).

2.5. Antibody reagents

An anti-rMSG1 immune serum (RαMSG1) was raised in rabbits as previously described [16]. Immunisations were conducted under the registration number 156/2002 with the legal prescriptions. Rabbit IgG was purified using a HiTrap ProteinA affinity column (GE Healthcare, Otelfingen, Switzerland). Rabbit IgG was labelled with Biotin using the Biotin Labelling Kit (Roche-Diagnostics).

2.6. SDS-PAGE and immunoblot analysis

SDS-PAGE and immunoblots were performed according to standard procedures [17,18]. Immunoblots were probed with *E. coli* absorbed sera from experimental piglets [15] and immunised rabbits. The purity of the *E. coli* membrane fractions was verified using a mouse monoclonal antibody to the RNA polymerase beta subunit of *E. coli* (Abcam, Cambridge, UK).

2.7. Mycoplasma membrane purification

M. suis membranes were isolated by osmotic lysis [19]. Briefly, *M. suis* was resuspended in 2 M glycerol and lysed with water. Samples were incubated at 37 °C for 15 min, and centrifuged (40,000 × *g*, 1 h). After washing, the membranes were resuspended in 500 µl of Beta buffer (7.5 mM NaCl, 2.5 mM Tris pH 7.4, 0.5 mM 2-mercaptoethanol), and concentrated over a 30–60% continuous sucrose gradient (40,000 × *g*, 12 h). The membrane band was extracted with a syringe.

2.8. Immunogold labelling of M. suis

Coagulated blood was fixed in 3% paraformaldehyde and embedded in LR White (Plano, Wetzlar, Germany). Grids with ultrathin sections were incubated with RαMSG1 and pre-immune serum at 4 °C overnight. Sections were then rinsed with 0.5% BSA and 0.1% gelatine in PBS and placed for 2 h on drops of anti-rabbit IgG gold conjugate (10 nm, 1:50, Sigma, Buchs, Switzerland). After staining with 1% uranyl acetate and 0.1% lead citrate the sections were examined using a Zeiss EM 900 transmission electron microscope at 75 kV.

2.9. MSG1 enzyme activity

To confirm the enzymatic activity of rMSG1, we measured the GAPDH activity using the Ferdinand assay [20]. Optical density at 340 nm was measured over a 90 s time span using a Shimadzu Spectrophotometer UV-160A (Shimadzu, Reinach, Switzerland).

2.10. Erythrocyte membrane preparation

Erythrocyte membranes were prepared by hypotonic lysis. Washed erythrocytes were lysed in 30 ml ice-cold lysis buffer (5 mM $NaPO_4$, 1 mM EDTA, pH 7.6). The membranes were sedimented at 31,000 × *g* for 15 min at 4 °C and resuspended in PBS. The protein content was determined using the Bio-Rad Protein Assay Kit (Bio-Rad, Reinach, Switzerland).

2.11. Binding of MSG1 to the erythrocyte membrane

Microtiter plates were coated with erythrocyte membranes or with BSA (negative control). Purified rMSG1 was added for 1 h at room temperature. After washing, bound protein was detected by using biotinylated RαMSG1 IgG and streptavidin–POD (Sigma). Wells coated with BSA did not exceed OD values of 0.1, and these background values were subtracted from individual samples. Inhibition of binding was monitored by pre-incubating rMSG1 with RαMSG1. Rabbit pre-immune serum and *E. coli* lysate served as negative controls.

2.12. Dot blot analysis

Surface localisation of MSG1 in *E. coli* was tested by dot blot. Induced and non-induced *E. coli_msg*1 were spotted onto a nitrocellulose membrane. MSG1 surface localisation was determined by using RαMSG1 (pre-adsorbed with *E. coli*). The intactness of the *E. coli* cells was verified using a monoclonal antibody to the RNA polymerase beta subunit from *E. coli*.

2.13. Microscopic examination of adhesion

Blood smears were incubated with *E. coli_msg*1*_gfp* or *E. coli_gfp* for 30 min. To eliminate unbound bacteria blood smears were washed five times with PBS. Adhesion was visualised under an Olympus BX40 fluorescence microscope. *M. suis* attachment to erythrocytes was visualised in acridine orange-stained blood smears from experimentally infected pigs [14].

2.14. Flow cytometry

The adhesion of rMSG1 expressing *E. coli* to erythrocytes was determined by FACS analysis. Pig erythrocytes bound on poly-D-lysine plates were incubated with 1 ml *E. coli_msg*1*_gfp* or *E. coli_gfp* for 1 h. After washing, the erythrocytes were resuspended in PBS. Inhibition assays were performed accordingly after pre-incubation of *E. coli* with rMSG1, RαMSG1, and control serum, respectively. With an FACScan flow cytometer (Becton Dickinson), erythrocytes were gated by forward- and side-scatter settings and the fluorescence channel 1 was used to quantify the fraction of erythrocytes with bound GFP expressing *E. coli*.

Surface accessibility of MSG1 on *E. coli* transformants was also proven by FACS analysis. *E. coli* cultures (*E. coli_msg*1, induced and non-induced) were resuspended in PBS to an $OD_{600\ nm}$ of 1.0 and washed three times. *E. coli* transformants were incubated with RαMSG1, FITC-labelled goat anti-rabbit antibodies (Sigma) and stained with the nucleic acid dye propidium iodide (Sigma) which enters only non-viable permissive bacterial cells.

2.15. Accession number

The sequence has been deposited in EMBL Nucleotide Sequence Database under accession number: AM407404.

3. Results

3.1. Identification of the putative adhesion protein MSG1

To identify pathways of *M. suis* adherence to porcine erythrocytes, genomic *M. suis* libraries were screened by shotgun sequencing. By means of sequence analysis and database alignments we identified clone SG1H10 containing an insert with homology to the *M. penetrans* GAPDH gene (*gap*; 64.3% identity in 737 nt overlap). Since GAPDH of several pathogenic bacteria reportedly possesses multiple binding activities [9,10,21], we selected the SG1H10 clone for further studies. Sequence analysis revealed that the library clone SG1H10 contains a 2067 bp insert with an average G + C content of 30.69%. Clone SG1H10 includes an open reading frame

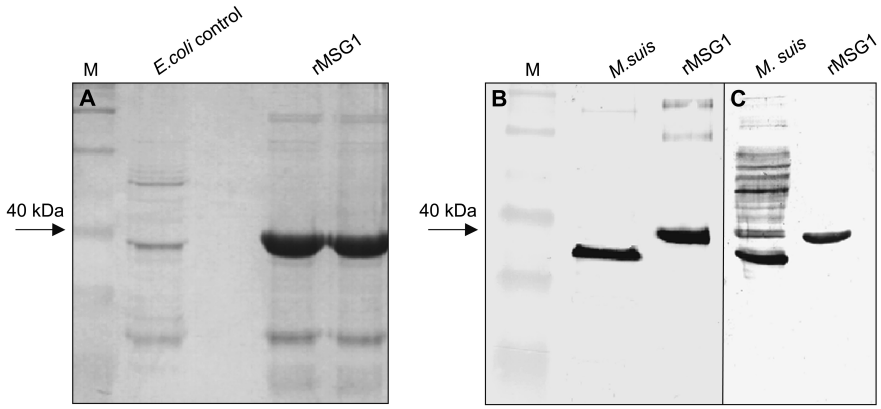

Fig. 1. (A) SDS polyacrylamide gel electrophoresis of recombinant *M. suis* MSG1. *E. coli* control, non-induced IMAC purified *E. coli* lysate; rMSG1 (two lanes), arabinose-induced, IMAC purified recombinant MSG1; and M, molecular weight marker. (B) Immunoblot analysis of recombinant MSG1 and *M. suis* whole cell antigen; immunological detection with an MSG1-specific rabbit polyclonal serum. (C) Immunoblot analysis of recombinant MSG1 and *M. suis* whole cell antigen; immunological detection with convalescent porcine serum pool.

(ORF) of 1011 bp which shows the highest homology to *M. penetrans gap* (63.32%). We designated this new gene *msg*1 (*M. suis* GAPDH-like protein 1). The gene *msg*1 translated into a protein of 336 amino acids with a predicted molecular mass of 36.7 kDa and an isoelectric point of 6.67. The predicted protein MSG1 displayed the highest degree of homology to GAPDH. In particular, the overall degrees of identity to GAPDH of *M. pneumoniae*, *M. genitalium*, and *M. penetrans* were calculated to be 54.5%, 53.3%, and 52.6%, respectively. The characteristic active site of GAPDH (amino acid sequence ASCTTNAL), which is responsible for the catalysis of the oxidative phosphorylation of D-glyceraldehyde-3-phosphate, was identified at MSG1 aa positions 157–164 using the program MOTIFS.

3.2. Expression and localisation of recombinant MSG1 (rMSG1) in E. coli

The entire ORF of the *msg*1 gene was modified and assembled as a synthetic gene to circumvent the inability to express *Mycoplasma* proteins in *E. coli* due to the atypical UGA codon usage. Induction of *E. coli_msg*1 resulted in the expression of a protein migrating on SDS-PAGE with an apparent molecular mass of 40 kDa (Fig. 1A). Recombinant MSG1 was used to raise an MSG1-specific rabbit polyclonal antiserum (RαMSG1). The specificity of RαMSG1 was demonstrated by probing an immunoblot containing rMSG1 and an *M. suis* preparation. Single bands of 40 kDa corresponding to the rMSG1 and the *M. suis* MSG1 were detected (Fig. 1B). The 2-kDa size shift between rMSG1 and *M. suis* MSG1 is caused by the 6-His-tag and *myc* epitope fused to rMSG1. The specific immunoreactivity of MSG1 was confirmed by immunoblot using a convalescent serum pool from experimentally infected pigs (Fig. 1C).

To determine the subcellular localisation of MSG1 in the *E. coli* transformants, the two cell compartments cytoplasm and membrane fraction were analysed by immunoblotting using RαMSG1. As shown in Fig. 2B a 40-kDa band, corresponding to the rMSG1, was found in both purified compartments. We used a monoclonal antibody to the RNA polymerase beta

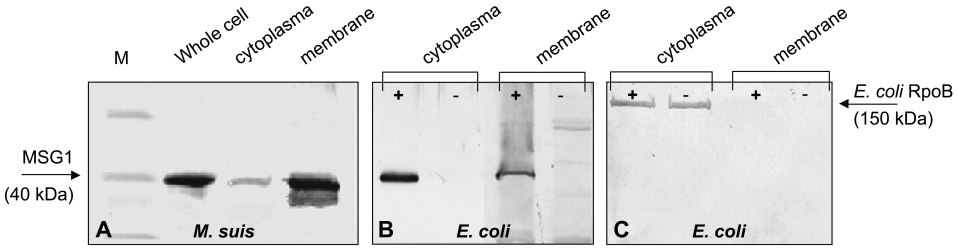

Fig. 2. (A) Immunoblot analysis of the subcellular localisation of MSG1 in *M. suis*. Lanes: *M. suis* whole cell preparation; *M. suis* cytoplasma fraction; *M. suis* membrane fraction; and M, molecular weight marker. Blot was reacted with RαMSG1. (B and C) Immunoblot analysis of the subcellular localisation of MSG1 in *E. coli* transformants. Lanes: +, arabinose-induced, MSG1 expressing *E. coli*; –, non-induced *E. coli* controls. (B) Blot was reacted with RαMSG1. (C) Blot was reacted with a monoclonal antibody against intracellular RNA polymerase beta subunit of *E. coli*.

subunit to reinforce the purity of the membrane fraction. The RNA polymerase antibody reacted only with the cytoplasmic proteins (Fig. 2C).

To investigate whether MSG1 is surface-exposed in *E. coli* transformants, we performed dot blot analyses on entire bacterial cells. *E. coli_msg*1 grown in the presence of arabinose reacted specifically with RαMSG1 (Fig. 3A) indicating the surface expression of MSG1 in *E. coli* transformants. No reaction could be observed using the monoclonal antibody against the *E. coli* RNA polymerase beta subunit confirming the intactness of the *E. coli* cells (data not shown). In contrast, *E. coli_msg*1 grown in the absence of arabinose showed no reactivity with RαMSG1. Surface exposition of rMSG1 was further confirmed by FACS analyses performed on whole cell *E. coli*. Arabinose-induced *E. coli_msg*1 were stained by RαMSG1 and a peak of fluorescence which is absent in non-induced controls was observed (Fig. 3B). The intactness of the native whole cell *E. coli_msg*1 was proven by propidium iodide staining (Fig. 3C).

3.3. MSG1 shows glycolytic activity

Recombinant MSG1 was assayed for GAPDH activity to confirm its functionality. Thereby, rMSG1 yielded a specific

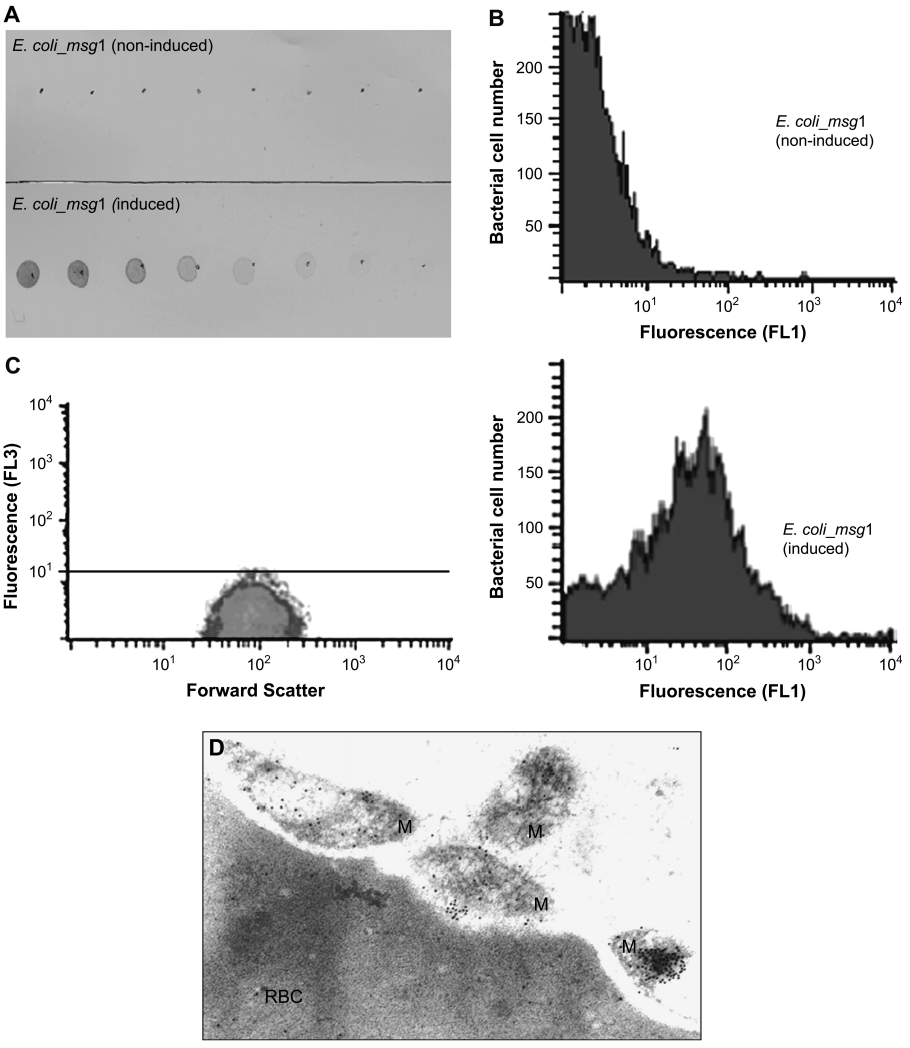

Fig. 3. Surface accessibility of MSG1. (A– C) On *E. coli* transformants: (A) dot blot analysis: induced and non-induced *E. coli_msg*1 whole cell transformants were spotted onto a nitrocellulose membrane and reacted with RαMSG1. (B) FACS analyses of the surface accessibility of MSG1 on *E. coli* transformants (histograms). Induced and non-induced *E. coli* whole cell transformants were stained with RαMSG1. (C) Intactness of the *E. coli* cells was confirmed by propidium iodide staining (dot plot diagram). Only *E. coli* cells not stained with propidium iodide were used for the analyses. (D) Immunogold transmission electron microscopy showing the surface accessibility of MSG1 in *M. suis*. Specific accumulation of 10 nm gold particles on *M. suis* (M) on the surface of a porcine erythrocyte (RBC).

activity of 73.9 μmol NADH min^{-1} mg^{-1}. The GAPDH activity measured for the *E. coli* control was significantly lower (21.9 μmol NADH min^{-1} mg^{-1}), and there was no detectable spontaneous conversion of NAD^+ to NADH without addition of DL-GAP to the reaction mixtures.

3.4. Localisation of MSG1 in M. suis

To localise MSG1 in *M. suis* cells, whole *M. suis* cells as well as the cytoplasmic and the membrane protein fractions were analysed for the presence of MSG1. RαMSG1 reacted with all three fractions, thus confirming the presence of intracellular located and membrane-associated MSG1 (Fig. 2A). To confirm the presence of MSG1 on the surface of *M. suis*, we performed immunoelectron microscopy with RαMSG1. Accumulation of gold labelled antibodies could be observed both, intracellular and on the surface of *M. suis* cells (Fig. 3D).

3.5. Adhesion of rMSG1 to porcine erythrocytes

Binding activity of rMSG1 to erythrocytes was assessed by ELISA. Purified rMSG1 was added at decreasing concentrations to RBC lysate. Specifically bound rMSG1 was detected with biotinylated RαMSG1. As shown in Fig. 4, purified rMSG1 binds to the RBC preparation in vitro in a dose-dependent manner. The rMSG1 binding was specifically blocked by RαMSG1.

3.6. MSG1 mediates adhesion of E. coli transformants to erythrocytes

Adhesion of *E. coli_msg*1*_gfp* was visualised by fluorescence microscopy. A specific adhesion of *E. coli_msg*1*_gfp* to porcine erythrocytes was clearly visible (Fig. 5). In contrast, the control strain *E. coli_gfp* showed no adhesion (Fig. 5).

Quantification of the adhesion of *E. coli* transformants was performed by FACS analyses. The results of the adhesion and adhesion inhibition assays are presented in Fig. 6. An average of $34.5\% \pm 2.9\%$ erythrocytes had attached *E. coli_msg*1*_gfp*. Adhesion of the same recombinants was significantly reduced in the presence of RαMSG1 and rMSG1 ($p \leq 0.05$): in the presence of RαMSG1 an average of $15.6\% \pm 3.2\%$ erythrocytes was loaded with *E. coli_msg*1*_gfp*, and in the presence of rMSG1 an average of $17.2\% \pm 3.8\%$ erythrocytes had attached *E. coli_msg*1*_gfp*. Pre-immune sera had only a poor reduction effect on the *E. coli_msg*1*_gfp* adhesion to erythrocytes. Control *E. coli_gfp* adhered to erythrocytes at levels nearly five times lower than *E. coli_msg*1*_gfp* ($7.1\% \pm 2.1\%$; $p \leq 0.05$).

4. Discussion

Attachment to host erythrocytes is essential for the unique life cycle of haemoplasma and an important initial event in the pathogenesis of their diseases. This in vivo interaction of *M. suis* with the erythrocytes is accompanied by irreversible deformations of the cell surface leading to a haemolytic anaemia in acute infected pigs [1,2]. To date, the molecular mechanisms responsible for the *M. suis* attachment have not been identified mainly due to numerous technical difficulties and limitations caused by the lack of an in vitro cultivation system.

Thus, we embarked on the strategy to screen genomic *M. suis* DNA libraries using a shotgun sequencing approach in order to determine putative *M. suis* adhesins. We were able to identify a complete ORF (*msg*1) which showed the highest homology (63.32%) to the GAPDH of *M. penetrans*. Adhesion properties of surface-localised GAPDHs have been described for many pathogens [21]. For example: GAPDH of *M. genitalium* has been reported to bind mucin [9], and GAPDH of group A streptococci has been noted to bind

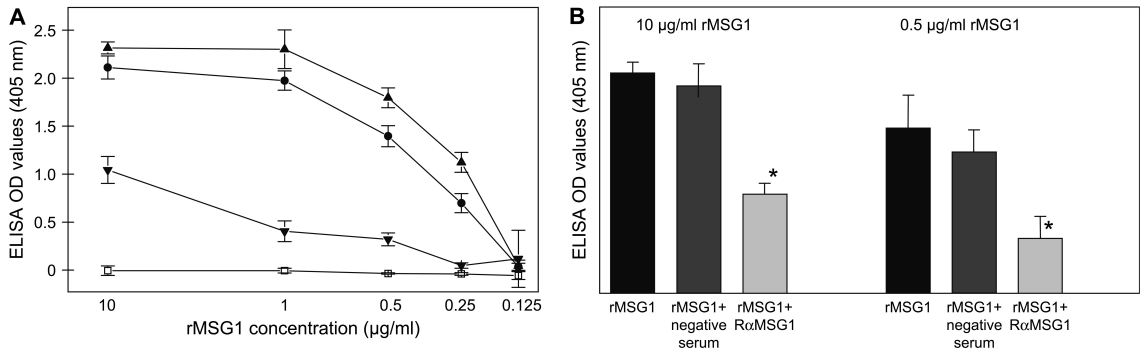

Fig. 4. (A) Binding of rMSG1 to erythrocyte lysate. Microtiter wells were coated with 10 μg (▲), 5 μg (●), and 1 μg (▼) of pig erythrocyte lysate, and decreasing concentrations of rMSG1 were added to individual wells. Bound protein was detected with biotinylated RαMSG1 and streptavidin–peroxidase. Values represent five independent assays. □, Values indicate negative control reactions (*E. coli* not expressing MSG1). (B) Inhibition of rMSG1 binding to immobilized erythrocyte lysate by antibodies to MSG1 (RαMSG1). Each bar represents the mean (plus standard deviation) of ELISA values from five experiments. *, $p \leq 0.05$ (one-way ANOVA).

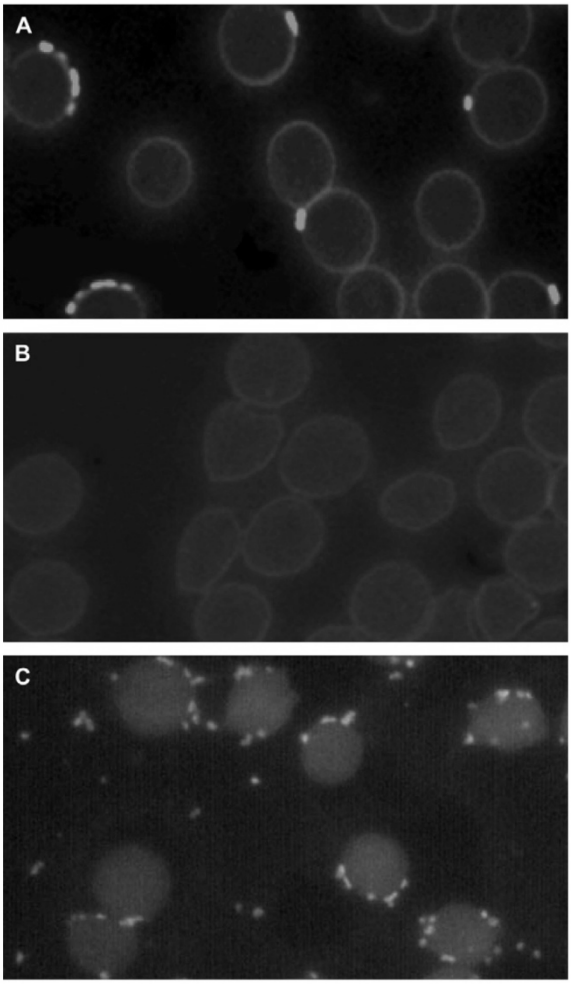

Fig. 5. Fluorescence microscopy showing adhesion to pig erythrocytes: (A) *E. coli_msg*1*_gfp* expressing MSG1 and GFP as fusion protein; (B) *E. coli_gfp* expressing GFP alone; and (C) *M. suis* attaching to pig erythrocytes in experimentally infected pigs (acridin orange-stained blood smear).

fibronectin, lysozyme, and the cytoskeletal proteins myosin and actin [10,21]. The binding activities were correlated in all studies with a function in the first step of infection, i.e. adhesion. Furthermore, these proteins were proven to show glycolytic activity [21].

One prerequisite of potential adhesion proteins is their surface accessibility. By means of immunoblot and immunoelectron microscopy we were able to locate MSG1 in *M. suis* in the cytoplasm, the membrane, and on the surface of *M. suis*. GAPDH is a key component of the glycolytic pathway. As such, the major functional location is cytoplasmic. However, it is known for many other pathogens that bacterial proteins with GAPDH properties are surface-localised so as to carry out a variety of accessory virulence associated functions [21]. The presence of MSG1 in the membrane and on the surface of *M. suis* is highly indicative of enzymatic as well as non-enzymatic functions of MSG1 in *M. suis* cells. But verifications on the actual biological role of MSG1 in *M. suis* are hampered by the unculturability of *M. suis* since only purified bacteria lysed from erythrocytes are available for functional analyses. Therefore, our strategy was to use MSG1 expressing *E. coli* transformants as a model system. In these *E. coli* transformants the localisation of MSG1 is identical to the localisation found in *M. suis*: in the cytoplasm, the membrane and on the surface. The absence of cytoplasmic components in the membrane preparation was verified by immunoblotting using a monoclonal antibody directed against the cytoplasmic RNA polymerase beta subunit [9]. Cytoplasmic rMSG1 showed GAPDH activity which in turn proved that rMSG1 is expressed in native conformation. Moreover, we assume that MSG1 could be etiologically involved in the development of severe hypoglycaemia based on the glycolytic metabolism of *M. suis* during clinical acute disease [12,13]. Surface-localised MSG1 seems to be involved in the RBC parasitism since non-adherent *E. coli* cells gain the ability to bind to erythrocytes by expression of MSG1 on their surface. This *E. coli_msg*1 adherence was significantly and specifically reduced by anti-MSG1 antibodies as well as by rMSG1. This renders proof that MSG1 is involved in the adhesion of *M. suis* to the host cell erythrocyte. In addition, the ubiquitous nature of MSG1 among different *M. suis* isolates along with its high sequence stability (data not shown) also reflects the common and perhaps important function of MSG1 for the organism.

Nevertheless, the fact that a protein showing GAPDH activity is expressed on the surface of gram-negative bacteria is rather curious, especially due to the fact that we found no classical signal peptide in the MSG1 sequence. Therefore, we verified the surface localisation of MSG1 in *E. coli* by two independent methodologies: FACS analysis and dot blot. At the moment we have performed detailed studies on the expression pathway of MSG1 in *E. coli* using different mutants.

In conclusion, we identified the 40-kDa *M. suis* protein MSG1 as a multifunctional protein showing enzymatic GAPDH activity as well as non-enzymatic properties, i.e. adhesion to the porcine erythrocytic surface. This report is the first to show a protein with potential virulence properties in the novel bacterial group of haemoplasmas. Our studies clearly showed that the heterologous expression system *E. coli* is well suited to carry out functional tests on proteins of unculturable hemotrophic mycoplasma.

Further studies are required to identify other membrane interactors that could be part of a potential adhesion complex of *M. suis*, and to identify erythrocyte receptors mediating the adhesion of haemoplasmas. Understanding the cellular interactions and factors that contribute to this complex relationship between pathogen and host is essential towards our ability to modulate its clinical outcomes. Furthermore, the identification of the adhesion protein MSG1 can not only provide a better understanding of the biology of *M. suis* but it can also help to rationally design vaccines or reagents used for the diagnosis of porcine *M. suis* infections.

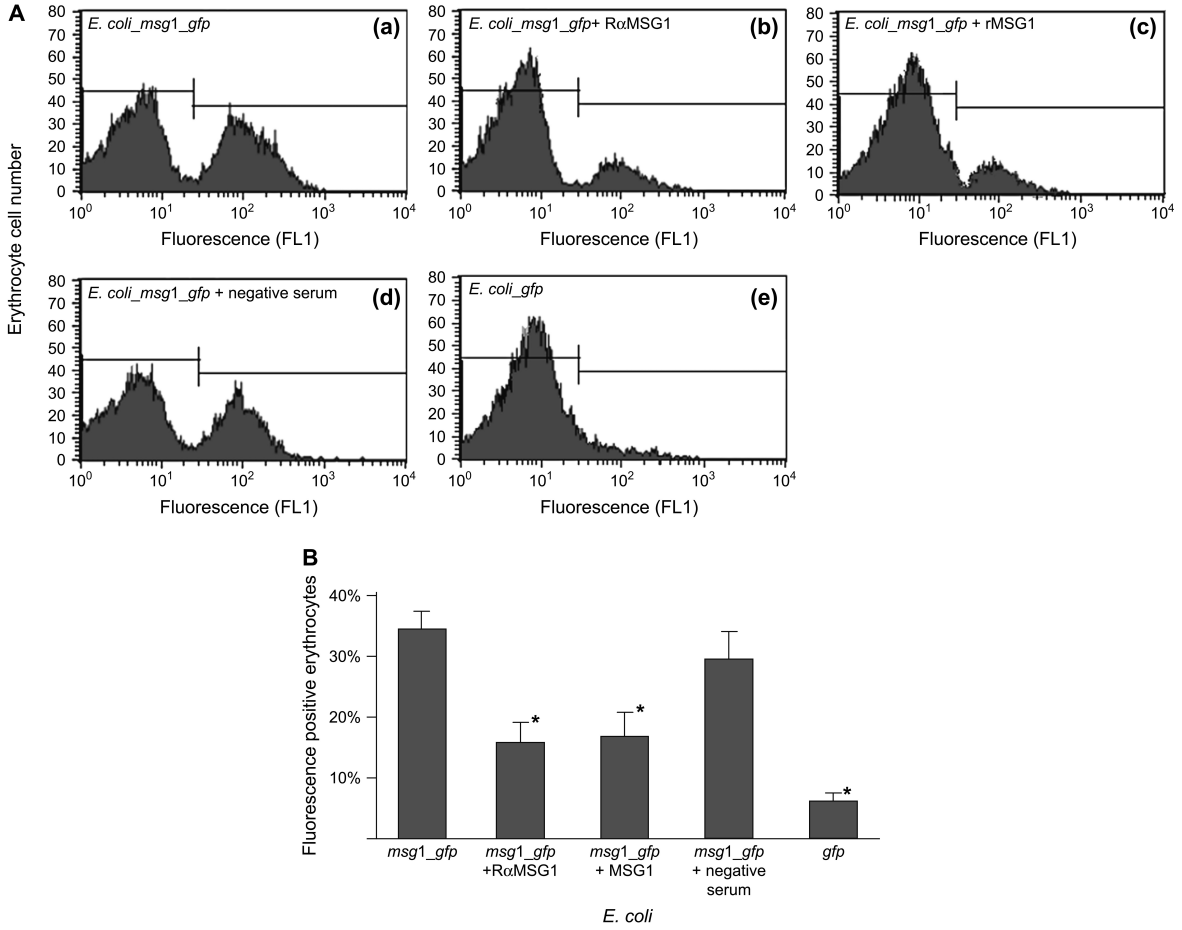

Fig. 6. Binding of *E. coli_msg*1 transformants to porcine erythrocytes. (A) Histograms of flow cytometer analyses. Binding to erythrocytes was visualised by the *E. coli_gfp* fluorescence. Erythrocytes were incubated with: (a) *E. coli_msg*1_*gfp*; (b) *E. coli_msg*1_*gfp* pre-incubated with antibodies to MSG1; (c) *E. coli_msg*1_*gfp* pre-incubated with rMSG1; (d) *E. coli_msg*1_*gfp* pre-incubated with pre-immune serum; and (e) *E. coli_gfp* without *msg*1. Line markers indicate the fluorescence line for gfp-positive cells. (B) Binding of *E. coli_msg*1_*gfp* to pig erythrocytes was significantly higher than the binding of *E. coli_gfp* without MSG1, and *E. coli_msg*1_*gfp* binding was significantly inhibited by antibodies to MSG1 (RαMSG1) and rMSG1 ($p \leq 0.05$). Each bar represents the mean (plus standard deviation) values from five FACS experiments. *, $p \leq 0.05$ (one-way ANOVA).

References

[1] J.B. Messick, Hemotrophic mycoplasmas (hemoplasmas): a review and new insights into pathogenic potential, Vet. Clin. Pathol. 33 (2004) 2–13.

[2] H. Neimark, K.E. Johansson, Y. Rikihisa, J.G. Tully, Proposal to transfer some members of the genera *Haemobartonella* and *Eperythrozoon* to the genus *Mycoplasma* with descriptions of 'Candidatus *Mycoplasma haemofelis*', 'Candidatus *Mycoplasma haemomuris*', 'Candidatus *Mycoplasma haemosuis*' and 'Candidatus *Mycoplasma wenyonii*', Int. J. Syst. Evol. Microbiol. 51 (2001) 891–899.

[3] A. Pospischil, R. Hoffmann, *Eperythrozoon suis* in naturally infected pigs: a light and electron microscopic study, Vet. Pathol. 19 (1982) 651–657.

[4] J.F. Zachary, E.J. Basgall, Erythrocyte membrane alterations associated with the attachment and replication of *Eperythrozoon suis*: a light and electron microscopic study, Vet. Pathol. 22 (1985) 164–170.

[5] J.F. Zachary, A.R. Smith, Experimental porcine eperythrozoonosis: T-lymphocyte suppression and misdirected immune responses, Am. J. Vet. Res. 46 (1985) 821–830.

[6] D.C. Krause, M.F. Balish, Structure, function, and assembly of the terminal organelle of *Mycoplasma pneumoniae*, FEMS Microbiol. Lett. 198 (2001) 1–7.

[7] S. Mudahi-Orenstein, S. Levisohn, S.J. Geary, D. Yogev, Cytadherence-deficient mutants of *Mycoplasma gallisepticum* generated by transposon mutagenesis, Infect. Immun. 71 (2003) 3812–3820.

[8] S. Razin, E. Jacobs, *Mycoplasma* adhesion, J. Gen. Microbiol. 138 (1992) 407–422.

[9] R.A. Alvarez, M.W. Blaylock, J.B. Baseman, Surface localized glyceraldehyde-3-phosphate dehydrogenase of *Mycoplasma genitalium* binds mucin, Mol. Microbiol. 48 (2003) 1417–1425.

[10] V. Pancholi, V.A. Fischetti, A major surface protein on group A streptococci is a glyceraldehyde-3-phosphate-dehydrogenase with multiple binding activity, J. Exp. Med. 176 (1992) 415–426.

[11] K. Heinritzi, G. Plank, W. Peteranderl, N. Sandner, The acid-base equilibrium and carbohydrate metabolism during infection with *Eperythrozoon suis*, Zentralbl. Veterinarmed. B 37 (1990) 412–417.
[12] J.E. Smith, J.E. Cipriano, S.M. Hall, In vitro and in vivo glucose consumption in swine eperythrozoonosis, Zentralbl. Veterinarmed. B 37 (1990) 587–592.
[13] N. Nonaka, B.J. Thacker, T.W. Schillhorn van Veen, R.W. Bull, In vitro maintenance of *Eperythrozoon suis*, Vet. Parasitol. 61 (1996) 181–199.
[14] L.E. Hoelzle, D. Adelt, K. Hoelzle, K. Heinritzi, M.M. Wittenbrink, Development of a diagnostic PCR assay based on novel DNA sequences for the detection of *Mycoplasma suis* (*Eperythrozoon suis*) in porcine blood, Vet. Microbiol. 93 (2003) 185–196.
[15] L.E. Hoelzle, K. Hoelzle, M. Ritzmann, K. Heinritzi, M.M. Wittenbrink, *Mycoplasma suis* antigens recognized during humoral immune response in experimentally infected pigs, Clin. Vaccine Immunol. 13 (2006) 116–122.
[16] L.E. Hoelzle, K. Hoelzle, M.M. Wittenbrink, Expression of the major outer membrane protein (MOMP) of *Chlamydophila abortus*, *Chlamydophila pecorum*, and *Chlamydia suis* in *Escherichia coli* using an arabinose-inducible plasmid vector, J. Vet. Med. B Infect. Dis. Vet. Public Health 50 (2003) 383–389.
[17] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[18] H. Towbin, T. Staehelin, J. Gordon, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, Proc. Natl. Acad. Sci. U.S.A. 76 (1979) 4350–4354.
[19] S. Razin, Cell lysis and isolation of membranes, in: S. Razin, J.G. Tully (Eds.), Methods in mycoplasmology, vol. 1, Academic Press, New York, 1983, pp. 225–233.
[20] W. Ferdinand, The isolation and specific activity of rabbit-muscle glyceraldehyde phosphate dehydrogenase, Biochem. J. 92 (1964) 578–585.
[21] V. Pancholi, G.S. Chhatwal, Housekeeping enzymes as virulence factors for pathogens, Int. J. Med. Microbiol. 293 (2003) 391–401.

8.4 Vaccination with the Mycoplasma suis recombinant adhesion protein MSG1 elicits a strong immune response but fails to induce protection in pigs

Vaccine. 2009; 27(39):5376-82.

Katharina Hoelzle, Susanne Doser, Mathias Ritzmann, Karl Heinritzi, Andreas Palzer, Sabine Elicker, Manuela Kramer, Kathrin M. Felder, Ludwig E. Hoelzle.

MSG1 and MSG1 expressing *E. coli* cells were used to vaccinate pigs prior to experimental infection with *M. suis*. A strong immune response was induced but failed to protect the animals from IAP. However, anaemia observed in *E.coli*_MSG1 vaccinated pigs was milder than in control animals.

My contribution was the analysis of splenocyte supernatants of IFN-γ concentration.

Vaccine 27 (2009) 5376–5382

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Vaccination with the *Mycoplasma suis* recombinant adhesion protein MSG1 elicits a strong immune response but fails to induce protection in pigs

Katharina Hoelzle[a,*], Susanne Doser[b], Mathias Ritzmann[c], Karl Heinritzi[b], Andreas Palzer[b], Sabine Elicker[b], Manuela Kramer[a], Kathrin M. Felder[a], Ludwig E. Hoelzle[a]

[a] *Institute of Veterinary Bacteriology, University Zurich, Winterthurerstr. 270, 8057 Zurich, Switzerland*
[b] *Clinic for Swine, Ludwig-Maximilians-University Munich, Sonnenstr. 16, 85764 Oberschleißheim, Germany*
[c] *Clinic for Swine, University for Veterinary Medicine, Veterinaerplatz I, 1210 Vienna, Austria*

ARTICLE INFO

Article history:
Received 5 May 2009
Received in revised form 10 June 2009
Accepted 22 June 2009
Available online 9 July 2009

Keywords:
Mycoplasma suis
Porcine infectious anemia
Adhesion protein MSG1
Vaccination

ABSTRACT

Mycoplasma suis is the unculturable pathogen of porcine infectious anemia. The study was aimed to determine the immunogenicity and protective efficacy of MSG1, an immunodominant adhesin of *M. suis* as the first vaccine candidate against *M. suis*. The results demonstrated that recombinant MSG1 and *Escherichia coli* transformants expressing MSG1 (*E. coli*_MSG1) induced a strong humoral and cellular immunity against *M. suis*. The induced antibodies were found to be functionally active as confirmed by an *in vitro* adhesion inhibition assay. Both, IgG1 and IgG2 antibodies were induced, but *E. coli*_MSG1 immune response was characterized by a significantly higher IgG1 antibody production. Both vaccine candidates failed to protect against *M. suis* challenge. However, *E. coli*_MSG1 vaccination has a considerable effect on the severity of the disease as shown by higher post-challenge hemoglobin and hematocrit values in comparison to control groups. This indicated that a high IgG1 antibody titer is negatively connected with severity of *M. suis*-induced anemia. Furthermore, the induction of monospecific anti-MSG1 antibodies by both vaccine candidates clearly allows for the differentiation between infected and vaccinated animals (DIVA principle). Overall, the importance of MSG1 as potential vaccine candidate remains to be established. Future studies will evaluate the conditions (i.e. adjuvant, vaccination scheme, and application route) to optimize the effects of *E. coli*_MSG1 vaccines.

1. Introduction

Mycoplasma suis is a hemotrophic, uncultivable pathogen in swine that causes acute hemolytic anemia and substantial economic losses in swine industry mainly due to chronic infections characterized by chronic anemia, general unthriftness, poor growth rates, and bad reproductive performance as well as predisposing the infected swine to secondary infections [1–6]. *M. suis* infections have been continuously reported worldwide over the past 75 years [2,7–12]. Substantial data on the prevalence of *M. suis* infections are limited due to the difficulties in diagnosing this uncultivable bacterium. However, current studies revealed a prevalence of about 10–15% in pig husbandry [4,18]. Traditionally, *M. suis* infection is controlled by the use of antibiotics and hygiene management (i.e. controlling bloodsucking insects; strict sanitary methods when vaccinating or castrating). However, this practice does not eliminate *M. suis* from infected pigs and persistently and clinically inapparent infected pigs remain carrier animals as well as potential *M. suis* transmitters within their herds or between herds. Therefore, in addition to the use of antibiotics and applying animal management procedures, the control and prevention of porcine infectious anemia through vaccination are needed.

To date, it has been impossible to develop a vaccination, because *M. suis* cannot be cultivated *in vitro*. Potential vaccine candidates would therefore have to be derived from the blood of clinically and acutely ill pigs. This, however, has at least two main restrictions: (i) vaccines contain porcine blood components with considerable side effects, i.e. an immune response against alloantigens, and (ii) the *M. suis* isolates are fully virulent and cannot be attenuated, for instance by means of culture passages. Furthermore, virulence factors of *M. suis* which could serve as vaccine candidates are not well defined. Obviously, the attachment to host erythrocytes is a crucial feature in the pathogenesis of *M. suis*-induced diseases [1,13,14]. *M. suis* is capable of colonizing and replicating on erythrocytes thereby damaging them and causing deformities. As shown by electron microscopy, *M. suis* and erythrocyte are connected by fibrillary attaching structures. The proteins that form these structures are, however, unknown. We recently identified the first *M. suis* adhesion protein (MSG1) which is highly conserved among different strains of *M. suis* [13]. The encoding gene *msg*1 was found in all

* Corresponding author. Tel.: +41 44 63 58635; fax: +41 44 63 58912.
E-mail address: khoelzle@vetbakt.uzh.ch (K. Hoelzle).

0264-410X/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2009.06.072

tested *M. suis* isolates. MSG1 is a surface-localized, immune dominant *M. suis* protein and porcine convalescent-phase sera have high titers of antibodies against this protein [9,15]. MSG1 was detected by all sera from experimentally infected pigs during the entire course of experimental infection (period of 16 weeks). Antibodies against MSG1 specifically inhibit the binding of recombinant MSG1 to erythrocyte lysate as well as the attachment of *Escherichia coli* transformants expressing MSG1 to porcine erythrocytes [13]. Therefore, MSG1 represents a promising target to develop effective vaccines against *M. suis*. In this study, we evaluated the efficacy of a vaccination against *M. suis* with recombinant MSG1 and *E. coli* transformants, expressing MSG1 in a porcine model. The immune response as well as protection performance of both vaccine candidates was thus determined and characterized.

2. Materials and methods

2.1. Bacteria and plasmids

The *M. suis* challenge strain 01/06 used in our study was taken from a pig suffering from acute infectious anemia. The strain was maintained in splenectomized pigs by subsequent experimental infection as described elsewhere [15,16]. For challenge infection EDTA-anti-coagulated blood was taken at maximum bacteremia (>80% of erythrocytes were infected) as confirmed by microscopic examination of acridine orange stained blood smears. Then, blood was stored at −80 °C until challenge experiment. The arabinose-inducible expression plasmid pBad*Myc*His (Invitrogen, Basel, Switzerland) was used to clone *msg*1. *E. coli* K12 LMG194 (Invitrogen), was used for the production of recombinant protein and immunization procedures and was cultivated in Luria-Bertani medium containing ampicillin (100 μg/ml) at 37 °C.

2.2. Preparation of vaccine candidates

Recombinant MSG1 (rMSG1) and MSG1-expressing *E. coli* transformants (*E. coli*_MSG1) were used as vaccine candidates. Culturing of *E. coli* transformants, and the expression and purification of MSG1 were performed as described previously [13,15]. MSG1 surface expression of *E. coli*_MSG1 was proven by dot blot analysis as described previously [13]. The protein content of preparations was determined using the Bradford assay (BioRad, Reinach, Switzerland). *E. coli* cultures were centrifuged and washed with PBS (Sigma, Buchs, Switzerland) and adjusted to 5×10^8 CFU/ml.

2.3. Immunization and challenge infection of pigs

A pig model was used to perform the immunization [15] and protection experiment using a protocol approved by the Government Office of the Upper Bavaria, Germany. Three-week-old pigs were screened for *M. suis*-negative status by quantitative PCR and *M. suis* immunoblot as described earlier [9,15,17]. The animal experiment included four groups of 10 *M. suis*-negative animals each: group A was vaccinated with 2.5 mg purified rMSG1 and group B was vaccinated with recombinant *E. coli*_MSG1 (5×10^8 CFU). Both antigens were mixed equally with incomplete Freund's adjuvant. Control groups C and D received PBS and non-recombinant *E. coli* (5×10^8 CFU), respectively, both mixed equally with incomplete Freund's adjuvant. Boost vaccination was performed 3 weeks apart. Blood samples were taken prior to the first (day 0) and second vaccination (day 21), and then once a week, until necropsy on day 84. On day 30, pigs were splenectomized according to methods described previously [16]. For challenge infection on day 35, 2 ml of the *M. suis* blood 01/06 (10^9 *M. suis*/ml blood) was administered intramuscularly. Clinical symptoms, feeding behavior, and body temperature of each individual pig were controlled at least at once a day. A scoring system was used to assess the clinical status of each pig. A score of 1 was given each for the occurrence of fever (body temperature >40 °C), lethargy, reduced food uptake, and pale/ear necrosis. The scores were added for each animal thus leading to maximal clinical scores of 4 per animal and day. Acutely diseased pigs with a clinical score of 4 were treated with tetracycline (40 mg/kg body weight) and glucose (35 g glucose/l drinking water). On day 84, pigs were euthanized. A pathological and histopathological examination procedure was conducted for all pigs.

2.4. Hematological and biochemical blood analysis

Hematological parameters were evaluated in EDTA-anti-coagulated blood using the Scil Vet ABC tool (Scil Animal Care Company GmbH, Viernheim, Germany) including hematocrit and hemoglobin values. Biochemical parameters, i.e. glucose and iron in blood sera were analyzed using the Hitachi 911 Chemistry Analyzer (Roche, Mannheim, Germany). Blood smears were stained with acridine orange and investigated under a fluorescence microscope (Olympus BX50; 1000-fold magnification).

2.5. Detection of *M. suis*-specific antibodies

In order to detect antibodies against *M. suis* immunoblotting and ELISA assays were performed as described earlier [9,15]. Recombinant MSG1 and HspA1, a surface-localized DnaK-analogous protein of *M. suis*, were used as *M. suis*-specific antigens [9]. Mouse anti-swine IgG1 and mouse anti-swine IgG2 (Cedi Diagnostics BV, Leylstad, The Netherlands) were used for IgG subtyping experiments. Prior to serological testing porcine sera were adsorbed using *E. coli* K12 LMG194.

2.6. Real-time PCR quantification of *M. suis*

For DNA extraction, 200 μl-volumes of whole anti-coagulated blood (EDTA) were mixed with equal volumes of lysis buffer (10 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 320 mM sucrose, 1% (v/v) Triton X-100) and centrifuged (8000 × *g*, 22 °C, 60 s). The pellet was suspended in 400 μl lysis buffer and then centrifuged again. After repeating this step once, the pellet was used to extract whole DNA with the GenElute Bacterial Genomic DNA Kit (Sigma).

M. suis DNA was detected and quantified with the LightCycler 2.0 System (Roche Diagnostics, Rotkreuz, Switzerland), as described previously [17]. For quantification purposes genomic *M. suis* DNA was extracted from experimentally infected pigs and *M. suis* was quantified, as described earlier [17].

2.7. Adhesion inhibition assay

Inhibition assay was performed as described previously with slight modifications [13]. Briefly, ELISA plates were coated with 500 ng/ml porcine erythrocyte membranes. Purified rMSG1 was used as an inhibition substance. Therefore, pig sera were pre-incubated with rMSG1 (100 ng/ml; 1 μg/ml; 5 μg/ml) at 37 °C for 1 h. The mixture was then added to the ELISA plates and incubated once again (room temperature, for 1 h). After washing, bound MSG1 protein was detected using biotinylated rabbit anti-MSG1 serum and streptavidin-POD (Sigma).

2.8. Lymphocyte proliferation assay

Pigs were splenectomized after the last immunization (day 30). A single spleen cell suspension was prepared. The erythrocytes were lysed with lysis solution (Becton Dickinson, Basel, Switzerland). Splenocytes were cultured in RPMI (Sigma) supplemented

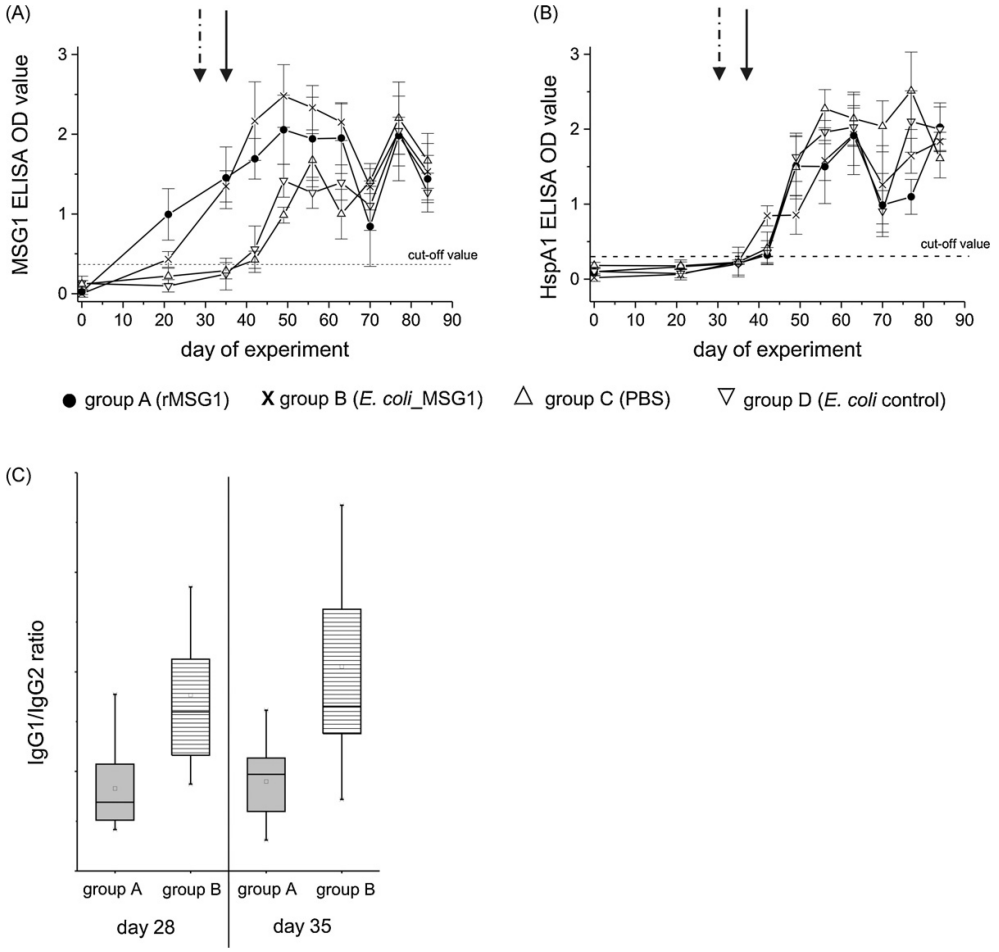

Fig. 1. Serum antibody response. Pigs were vaccinated with rMSG1, *E. coli*-MSG1 transformants, *E. coli* negative control and PBS on days 0 and 21. On day 35 pigs were splenectomized and challenged with a virulent *M. suis* isolate. Serum samples were collected at 1-week interval and antibody titers were measured by ELISA using rMSG1 and rHspA1 as antigens [9]. The absorbance values are means ± standard deviation. Arrows indicate the points of splenectomy (dashed arrow) and challenge infection (black arrow). (A) Antibody response against rMSG1; (B) antibody response against rHspA1; (C) analysis of IgG1 and IgG2 antibodies against rMSG1. The IgG1/IgG2 ratio was calculated from sera collected at days 28 and 35 post-vaccination (pre-challenge).

with 2 mM glutamine, 5% fetal bovine serum, and 10 μg/ml gentamicin sulfate at 37 °C with 5% CO_2 in 96-well cell culture plates at a concentration of 5×10^5 cells/well. Proliferation stimulation was performed using the following antigens: 1 μg/well of purified *M. suis* antigen [15], 1 μg/well non-infected blood preparation, 0.5 μg/well rMSG1, 0.5 μg/well non-induced purified *E. coli* control, or 2 μg Concavalin A (ConA). Splenocytes were cultured for 72 h and then treated with 10 μM BrdU (Roche Diagnostics) for 2 h. Incorporation of BrdU was measured using the BrdU Proliferation Assay Kit (Roche Diagnostics) according to the manufacturer's recommendations.

2.9. Statistical analysis

Data were compiled and analyzed with Excel for Windows (Microsoft, Wallisellen, Switzerland), Analyse-it (standard edition Software ONE AG), and Origin software (Redacom AG Nidau, Switzerland). The Wilcoxon test was used to assess differences with regard to antibody response and bacterial load. Differences were considered to be significant, if the *P* value was ≤0.05.

3. Results

3.1. Vaccination of pigs

3.1.1. Humoral immunity

Recombinant MSG1 (rMSG1) and MSG1-expressing *E. coli* transformants (*E. coli*_MSG1) were used as vaccine candidates. *M. suis*-negative pigs ($n = 40$) were assigned to four groups A–D as follows: group A was vaccinated with rMSG1, group B received *E. coli*_MSG1 transformants; in the negative control group C and D animals were immunized with PBS (group C) and non-recombinant *E. coli* (group D), respectively. MSG1-specific antibody response was analyzed from serum samples taken on day 0 (prior to immunization), 21, and 35 (prior to challenge). As shown in Fig. 1A all pigs reacted negative in the MSG1 ELISA prior to immunization. Following this, group A pigs showed a positive anti-MSG1 antibody response, even after the first immunization. The ELISA OD values increased distinctly after the boost immunization. In contrast, the initial immunization with the *E. coli*_MSG1 transformants led to positive but lower anti-MSG1 antibody titers. Following

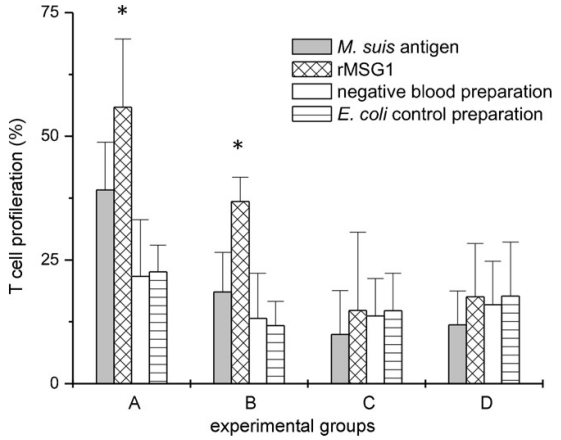

Fig. 2. Proliferation response of splenic lymphocytes post-vaccination (day 35) after specific stimulation (*M. suis* blood preparation and rMSG1) and unspecific proliferation (negative blood preparation and *E. coli* control). The Concavalin A proliferation rate was set as 100%. Bars represent mean values (%) plus standard deviation from all spleens of one group. Differences of proliferation rates after specific stimulation and after unspecific stimulation were significant (*) when $P < 0.05$.

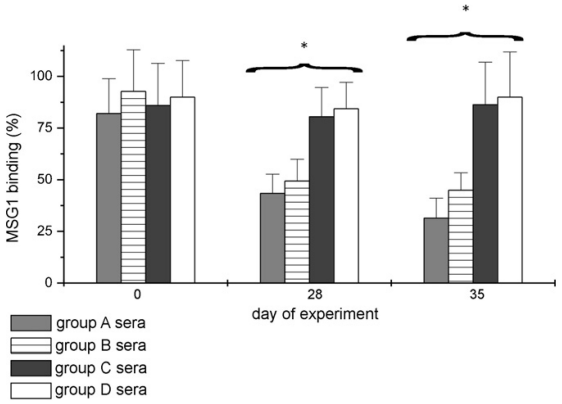

Fig. 3. Adhesion inhibition assay. Microtiter plates were coated with 500 ng/ml porcine erythrocyte lysate. The binding of rMSG1 to the immobilized erythrocyte lysate without inhibition was set 100%. Sera ($n = 10$) from all four groups were tested prior to immunization (day 0), after the first immunization (day 28), and after the second immunization (day 35). Sera from group A and B pigs inhibited significantly the binding of rMSG 1 (*$P < 0.05$), sera from the control groups C and D showed no inhibitory effect.

the boost immunization (day 21), the immunized pigs in group B demonstrated also very high positive anti-MSG1 antibody levels (Fig. 1). Anti-MSG1 antibodies of group A and B animals were detectable up until the end of the experiment (day 84). In the control groups C and D no positive anti-MSG1 antibody responses could be observed until day 35. Anti-MSG1 antibodies were only found in the sera of group C and D animals after challenge infection (day 42).

In order to investigate the type of the induced immune response MSG1-specific IgG subclasses IgG1 and IgG2 from all animals were measured after the first and the second immunization (on days 28 and 35). Basically, both IgG subclasses were induced in rMSG1 (group A) and *E. coli*_MSG1-immunized (group B) pigs. In the mock-vaccinated pigs of group C and D IgG1 and IgG2 values remained at baseline levels. Immunization with *E. coli*_MSG1 led to a significantly higher IgG1 level (43.55% IgG1/56.45% IgG2) compared to rMSG1 immunization (29.98% IgG1/70.02% IgG2; $P < 0.05$). Accordingly, the analysis of the subtype ratio revealed significantly higher IgG1/IgG2 ratio in pigs after MSG1-*E. coli* immunization on day 21 as well as on day 35 compared to the rMSG1 immunized pigs (Fig. 1C).

3.1.2. Cellular immunity

The antigen-specific lymphocyte proliferation was analyzed in order to determine the cellular immune response. Splenic lymphocytes from all animals were prepared after splenectomy, cultivated and finally stimulated with rMSG1 and purified *M. suis* preparation. Control preparations of non-induced *E. coli* cultures and blood derived from non-infected pigs were applied as a negative control. The proliferation rate following stimulation with the mitogen Concavalin A was set at a 100% rate, thus allowing for the determination of the proliferation rate (%) after stimulation with specific and control antigens. For the vaccinated groups A and B, stimulation with rMSG1 and *M. suis* preparations led to a significantly higher stimulation rate in comparison to the control preparations ($P < 0.05$). Within the control groups C and D no significant difference in the proliferation rate following specific stimulation (rMSG1 and *M. suis*) and unspecific stimulation (*E. coli* control, negative blood preparation) could be made out. Fig. 2 shows detailed proliferation data.

3.2. Protective efficacy

3.2.1. Protective efficacy in vitro (adhesion inhibition assay)

To further evaluate the potential of MSG1 as a vaccine, we tested whether anti-MSG1 antibodies induced by immunization with rMSG1 and *E. coli*_MSG1 transformants were capable of inhibiting the adhesion of MSG1 to porcine erythrocytes. Prior to binding to porcine erythrocyte lysate, rMSG1 was pre-incubated with sera from vaccinated as well as control animals. As illustrated in Fig. 3 groups A and B sera taken after the boost immunization showed a significant inhibition of MSG1 binding ($P < 0.05$) in a dose dependent manner. Contrary to this, no significant inhibitory effects could be observed in sera from group C and D pigs.

3.2.2. Protective efficacy in vivo (challenge infection in pigs)

M. suis challenge infection based on a splenectomized pig model leads to typical symptoms, i.e. clinically apparent anemia with maximum bacteremia (>80% infected erythrocytes) at intervals of 7 days. At the onset of the attack the animals were treated with tetracycline and glucose to prevent death by *M. suis*-induced hypoglycemia. Using a scoring system to assess the clinical status of animals (fever, lethargy, reduced food uptake, and pale/ear necrosis), no significant differences could be observed between the vaccinated animals (groups A and B) and the control animals (data not shown). In each group two to five animals had to be euthanized before the end of the experiment period due to their poor health condition. Therefore, no statistical analysis could be carried out. Anemia-associated blood parameters (i.e. hematocrit, hemoglobin, iron and glucose values) served to evaluate the severity of the disease. A considerable decrease in hematocrit and hemoglobin values could be determined in all experimental groups from day 42 (Fig. 4). No differences could be made out between the rMSG1-vaccinated group (group A) and the control groups. However, between days 42 and 56 the *E. coli*_MSG1 group (group B) showed substantially higher hematocrit and hemoglobin values than the control groups. Glucose and iron values decreased considerably 1 week after the challenge infection (day 42). For both parameters there was no difference between vaccinated and non-vaccinated pigs.

During the challenge infection, the *M. suis* blood concentration was tested by means of quantitative PCR (Fig. 5). Three days after the challenge infection, *M. suis* was found in the blood of all animals.

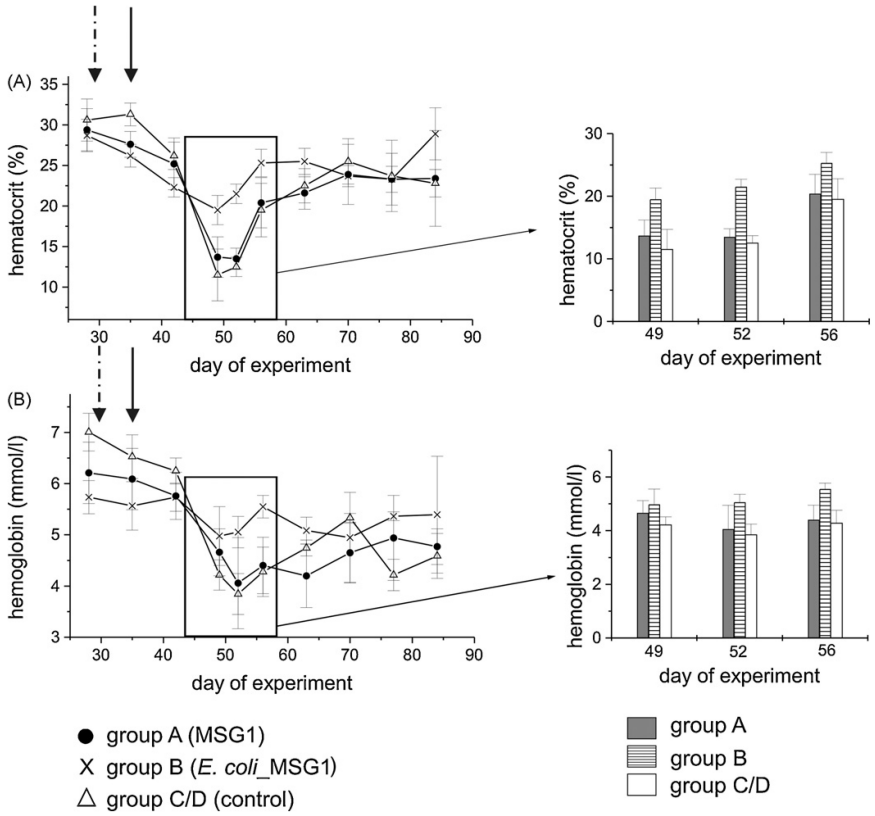

Fig. 4. Hematocrit (A) and hemoglobin (B) values of the four vaccination groups during the challenge infection. Control groups C and D were summarized in one group. Arrows indicate the points of splenectomy (dashed arrow) and challenge infection (black arrow). Boxes indicate the challenge period from day 49 to day 56 which was illustrated as bar diagram on the right. Values represent means of the vaccination groups ± standard deviation.

From day 42 onwards, the *M. suis* load increased considerably in all animals. In comparison to the control groups C and D, group A showed no differences. In comparison to the control groups, group B animals, however, demonstrated a substantially lower *M. suis* load 2 weeks p.i. (day 56). In this group, the *M. suis* loads only increased to the level of the control groups again as of day 77.

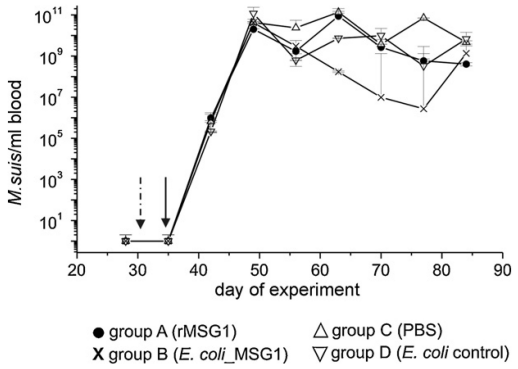

Fig. 5. Kinetics of the bacterial load of the blood following challenge infection. Quantification was performed using a quantitative PCR [17]. Arrows indicate the points of splenectomy (dashed arrow) and challenge infection (black arrow). Values represent means ± standard deviation.

The pathological examination showed no differences between the groups. Post-mortem examinations of all groups showed a pathological picture of a high-grade anemia with icterus and cyanoses at the ears.

3.3. Differentiating vaccinated animals from infected animals

In addition to the protective efficacy of rMSG1 the potential of two recently developed recombinant ELISAs (MSG1-based and HspA1-based) [9] was investigated in order to differentiate vaccinated from infected animals. During the vaccination period, anti-MSG1 antibody values, as assessed in the rMSG1 ELISA (Fig. 1A), increased in the immunized groups A and B—whereas ELISA results in the control groups C and D remained negative. Antibody titers in the rHspA1 ELISA remained negative before the challenge in all groups. One week after the challenge, HspA1 seroconversion was observed in both—the vaccinated and the control groups. HspA1 antibody values remained positive until the end of the experiment (Fig. 1B; day 84). Consequently, a serological differentiation between the infected and the vaccinated animals was possible.

4. Discussion

Mycoplasmas depend on the metabolic power of eukaryotic cells. This characteristic leads to an intimate connection to these cells [18,19]. For *M. suis* the attachment to erythrocytes is essen-

tial for its unique life cycle and the pathogenesis of the induced disease [2,20]. Therefore, vaccines inducing an immune response against adhesion structures should prevent colonization of porcine erythrocytes by *M. suis*. However, due to its unculturability, very little is known about the adhesion determinants of these bacteria. To date, only one *M. suis* adhesion protein MSG1 has been identified and characterized [13]. This adhesion protein fulfills important prerequisites for the development of effective vaccines: MSG1 is surface-localized, conserved and a major immunogen of *M. suis* [13]. *M. suis* infected animals (both experimentally and naturally infected animals) develop high antibody titers against MSG1 [9,15].

In the present study, rMSG1 and *E. coli*_MSG1 transformants were evaluated (i) for their potential to induce MSG1-specific B-cell and T-cell responses in pigs, and (ii) for their protective efficacy in challenge trials. Both vaccine candidates strongly induced the production of anti-MSG1 antibodies and antibody titers could be boosted by a second vaccine application. The induced antibodies of both vaccine candidates were shown to be functionally active in an *in vitro* inhibition assay, i.e. the anti-MSG1 antibodies were able to inhibit the adhesion of MSG1 to porcine erythrocytes. For other porcine *Mycoplasma* infections it has been suggested that an effective immunity requires both, humoral and cell-mediated immune responses [21–23]. In our study we detected a significant MSG1-specific splenocyte proliferation response after vaccination with both vaccine candidates.

Because a protective effect was to be expected due to the immune response induced by vaccination (functionally active antibodies and cell-mediated immune response), we performed a challenge infection. An established splenectomized pig model was used despite the fact that these animals might not represent what might happen in intact pigs. Vaccination, however, was performed prior to splenectomy in order to not impair the immune response induced by the vaccine. Splenectomy induces a partial immunosuppression leading to an increase of the intensity of all the symptoms of *M. suis*-induced anemia characterized by acute clinical attacks in 7-day-intervals [14,15]. The effectiveness of an anti-*M. suis* vaccine can be evaluated by the determining of the clinical status as well as anemia-associated parameters, e.g. hemoglobin and hematocrit values.

In this study, however, the strong antibody response to rMSG1 and *E. coli*_MSG1 did not provide protection against challenge infection: all experimental groups, independent of their vaccination status, demonstrated acute anemia, and in part, critical states of health. The evaluation of clinical parameters by means of the score system revealed no difference among the groups. Unfortunately, animals from all groups had to be euthanized because of acute disease symptoms (animal welfare). Consequently, a statistical analysis of clinical parameters could not be performed because only a minimal number of animals were used for ethical reasons. Nevertheless, in comparison to control groups C and D, group B (vaccinated with *E. coli*_MSG1 transformants) showed considerable differences in the anemia-associated parameters, i.e. hemoglobin and hematocrit. We determined similar results regarding the *M. suis* blood load: group B demonstrated a considerably lower amount of *M. suis* than the control groups. Vaccination with rMSG1 in group A, however, did not bring about an improvement of the blood parameters or any reduction of the bacterial load in the blood.

When comparing the vaccination groups A and B we discovered only one significant difference: namely that the IgG subclass profile differed significantly. While both IgG1 and IgG2 subclasses were induced by both vaccine candidates the IgG1 response was higher in vaccination group B which was also demonstrated by the significantly higher IgG1/IgG2 ratio. Therefore, the IgG1 antibody titer was negatively correlated to the severity of the anemia. The protective efficacy of an antigen depends upon the type of immune response. The IgG subclass produced as a consequence of immunization reflects the type of immune responses (Th1/Th2-type). Indeed, the balance between Th1 and Th2 immune response is an important determinant for the protection against a given pathogen. In mice, IgG1 indicates a Th2-type immune response, whereas IgG2a is predominantly produced during a Th1-type response [24]. For pigs, however, the function and significance of the IgG1/IgG2 ratio for the Th1/Th2 balance are not well documented. It is only known from other studies, that higher IgG1 titers are crucial for the vaccine protection of pigs against bacterial and parasitic infections, i.e. *Actinobacillus pleuropneumoniae* [25] and *Trichinella spiralis* [26]. The results of the present study support the view that in pigs protective capacities of vaccines are enhanced by optimal vaccine inducing IgG1 antibodies as well as IgG1-directing adjuvant. Apparently, the application of MSG1-expressing *E. coli* transformants pushes the immune system towards an IgG1 dominated immune response possibly due to conformationally correct expression of the vaccination antigen on the surface of a heterologous host.

In principle, vaccination is the only possibility to control successfully and eradicate *M. suis* infections because antibiotic therapies (e.g. with tetracycline) of *M. suis*-induced diseases only improve the clinical symptoms, i.e. anemia and fever. The bacterium, however, cannot be eliminated [1]. Thus, infected animals remain chronically persistent infected hosts, show increased susceptibility towards multicausal intestinal and respiratory tract infections, and are the source of infection for other animals [2,10]. An ideal vaccine for livestock should be highly protective, safe, inexpensive, easy to use, and facilitate differentiation between infected and vaccinated animals (DIVA principle). With regard to an *M. suis* infection, currently due to the lack of defined culture-derived bacteria, this goal can only be reached with recombinant vaccines. The vaccination with both antigens (rMSG1 and *E. coli*_MSG1) induced a strong but not a protective immune response that enabled the differentiation between infected and vaccinated animals. Test results also showed that vaccination with *E. coli*_MSG1 led to a reduction of clinical symptoms, which suggests that this strategy could represent a new approach toward the design of vaccines against *M. suis*. However, further studies are necessary to improve substantially the vaccination approach and must involve the extension of the vaccines with new adhesion proteins and virulence factors as well as optimal adjuvant to promote significantly the IgG1-type immune response.

References

[1] Hoelzle LE. Haemotrophic mycoplasmas: recent advances in *Mycoplasma suis*. Vet Microbiol 2008;130(3–4):215–26.
[2] Messick JB. Hemotrophic mycoplasmas (hemoplasmas): a review and new insights into pathogenic potential. Vet Clin Pathol 2004;33:2–13.
[3] Henry SC. Clinical observations on eperythrozoonosis. J Am Vet Med Assoc 1979;174(6):601–3.
[4] Brownback A. Eperythrozoonosis as a cause of infertility in swine. Vet Med Small Anim Clin 1981;76:375–8.
[5] Schweighardt H, Fellner A, Pechan P, Leuermann E. Eperythrozoonose beim Schwein—ein Fallbericht. Wien Tierarztl Mschr 1986;73:250–3.
[6] Zinn GM, Jesse GW, Dobson AW. Effect of eperythrozoonosis on sow productivity. J Am Vet Med Ass 1983;182:369–71.
[7] Dipeolu OO, Majaro OM, Akinboade OA, Nwufor KJ. Studies on the blood parasites of pigs in Ibadan, Nigeria. Vet Parasitol 1982;10:87–90.
[8] Guimaraes AM, Biondo AW, Lara AC, Messick JB. Exploratory study of *Mycoplasma suis* (*Eperythrozoon suis*) on four commercial pig farms in southern Brazil. Vet Rec 2007;160:50–3.
[9] Hoelzle K, Grimm J, Ritzmann M, Heinritzi K, Torgerson P, Hamburger A, et al. Use of recombinant antigens to detect antibodies against *Mycoplasma suis*, with correlation of serological results to hematological findings. Clin Vaccine Immunol 2007;14:1616–22.
[10] Ritzmann M, Grimm J, Heinritzi K, Hoelzle K, Hoelzle LE. Prevalence of *Mycoplasma suis* in slaughter pigs, with correlation of PCR results to hematological findings. Vet Microbiol 2009;133(1–2):84–91.
[11] Schuller W, Heinritzi K, Al-Nuktha S, Kölbl S, Schuh M. Serologic progression studies using CF and ELISA for the detection of antibodies against *Eperythrozoon suis* infection of swine. Berl Munch Tierarztl Wschr 1990;103:9–12.
[12] Wu J, Yu J, Song C, Sun S, Wang Z. Porcine eperythrozoonosis in China. Ann N Y Acad Sci 2006;1081:280–5.

[13] Hoelzle LE, Hoelzle K, Helbling M, Aupperle H, Schoon HA, Ritzmann M, et al. A surface-localised protein of *Mycoplasma suis* is involved in the adhesion to erythrocytes. Microbes Infect 2007;9(4):466–74.
[14] Zachary JF, Smith AR. Experimental porcine eperythrozoonosis: T-lymphocyte suppression and misdirected immune responses. Am J Vet Res 1985;46:821–30.
[15] Hoelzle LE, Hoelzle K, Ritzmann M, Heinritzi K, Wittenbrink MM. *Mycoplasma suis* antigens recognized during humoral immune response in experimentally infected pigs. Clin Vaccine Immunol 2006;13(1):116–22.
[16] Heinritzi K. A contribution on splenectomy in swine. Tierarztl Prax 1984;12(4):451–4.
[17] Hoelzle LE, Helbling M, Hoelzle K, Ritzmann M, Heinritzi K, Wittenbrink MM. First LightCycler real-time PCR assay for the quantitative detection of *Mycoplasma suis* in clinical samples. J Microbiol Methods 2007;70:346–54.
[18] Nicolet J. Animal mycoplasmoses: a general introduction. Rev Sci Tech 1996;15(4):1233–40.
[19] Razin S, Yogev D, Naot Y. Molecular biology and pathogenicity of mycoplasmas. Microbiol Mol Biol Rev 1998;62(4):1094–156.
[20] Zachary JF, Basgall EJ. Erythrocyte membrane alterations associated with the attachment and replication of *Eperythrozoon suis*: a light and electron microscopic study. Vet Pathol 1985;22:164–70.
[21] Haesebrouck F, Pasmans F, Chiers K, Maes D, Ducatelle R, Decostere A. Efficacy of vaccines against bacterial diseases in swine: what can we expect? Vet Microbiol 2004;100(3–4):255–68.
[22] Okamba FR, Moreau E, Cheikh Saad Bouh K, Gagnon CA, Massie B, Arella M. Immune responses induced by replication-defective adenovirus expressing the C-terminal portion of the *Mycoplasma hyopneumoniae* P97 adhesin. Clin Vaccine Immunol 2007;14(6):767–74.
[23] Thacker EL, Thacker BJ, Kuhn M, Hawkins PA, Waters WR. Evaluation of local and systemic immune responses induced by intramuscular injection of a *Mycoplasma hyopneumoniae* bacterin to pigs. Am J Vet Res 2000;61(11):1384–9.
[24] Stevens TL, Bossie A, Sanders VM, Fernandez-Botran R, Coffman RL, Mosmann TR, et al. Regulation of antibody isotype secretion by subsets of antigen specific helper T cells. Nature 1988;334(6179):255–8.
[25] Furesz SE, Wilkie BN, Mallard BA, Rosendal S, MacInnes JI. Anti-haemolysin IgG1 to IgG2 ratios correlate with haemolysin neutralization titres and lung lesion scores in *Actinobacillus pleuropneumoniae* infected pigs. Vaccine 1998;16(20):1971–5.
[26] Serrano FJ, Perez-Martin JE, Carron A, Navarete I. Comparison of IgM, IgG1 and IgG2 responses to *Trichinella spiralis* and *Trichinella britovi* in swine. Parasite 2001;8:133–5.

9. Acknowledgements

During my Ph.D. studies I was supported by many people.

First, I would like to thank PD Dr. L. E. Hölzle for supervising my Ph.D. thesis. He always had great ideas and knew solutions for all problems. The same is true for his wife, Dr. Katharina Hölzle. Both of you supported and helped me with everything during the last years. I am very grateful for your trust and friendship.

Prof. M. M. Wittenbrink, the head of the Institute for Veterinary Bacteriology, is acknowledged for giving me the opportunity to perform my Ph.D. studies at his institute. He always was interested in my work and contributed with many fruitful inputs.

I wish to thank Prof. Dr. L. Eberl for acting as responsible faculty member and as head of my promotion committee and Prof. J. Pernthaler for attending the committee.

PD Dr. Kathrin Riedel is acknowledged for our cooperation project and help with the proteomic studies. I thank Dr. Paula Carranza, Dr. Peter Gehrig, Dr. Simon Barkow, Dr. Bernd Roschitzki and Dr. Stefan Schauer for the great support in the FGCZ lab.

I thank Manuela Kramer for doing all quantitative PCRs, Katrin Gröbel for scanning electron- and confocal laser scanning micrographs, Sabrina Schreiner for library clone sequence comparisons to my proteomic results, Anja Hamburger for many ELISAs and Hanspeter Rutschi for help with the pigs. All other staff is acknowledged for creating a good, friendly atmosphere at the institute.

The second part of my Ph.D. study time was financially supported by the „Forschungskredit“ of the University of Zurich.

Above all I thank my family and Michael for their support during the last years. Without them this never had been possible.

10. CV and List of Publications

Name	Kathrin M Felder
Title	Dipl. Natw. ETH
Date of birth	March 19, 1981
Nationality	Swiss
Current position	Ph.D. student
Email	kathrin.felder@vetbakt.uzh.ch

Education

1993 – 2000	general qualification for university entrance, Kantonsschule Beromünster.
2000 – 2003	undergraduate studies in general biology and biochemistry, Swiss Federal Institute of Technology, Zurich (ETHZ).
2003 – 2005	Graduate studies in microbiology and Diploma in Microbiology, Swiss Federal Institute of Technology, Zurich (ETHZ). Diploma thesis: “A DNA microarray for identification of *Bacillus anthracis* in environmental samples”. Supervisors: Prof. M. M. Wittenbrink and PD L.E. Hölzle
2006 – 2010	post graduate studies and position as Ph.D. student at the Institute for Veterinary Bacteriology, Vetsuisse Faculty, University of Zurich, Switzerland. Thesis: Analysis of the pathogenesis of severe anaemia of pigs due to *Mycoplasma suis* infection. Supervisor: PD Dr. L.E. Hölzle

Grants

2008 – 2010	Financial support by Forschungskredit of University of Zurich Project: Untersuchungen zur Pathogenese der hämolytischen Anämie während der *Mycoplasma suis*-Infektion des Schweines.
2008	Travel grant by the International Organization for Mycoplasmology (IOM) for attending the 17th IOM congress in Tianjin, China.
2008	Travel grant by the science faculty, University of Zurich for attending the 12th International Symposium on Microbial Ecology (ISME) in Cairns, Australia.

List of publications

Felder KM, Hoelzle K, Heinritzi K, Ritzmann M and Hoelzle LE (2010): Autoantibodies to actin in autoimmune haemolytic anaemia. *BMC Vet Res*, under review.

Hoelzle K, Peter S, Kramer MM, Wittenbrink MM, **Felder KM** and Hoelzle LE (2010): Inorganic pyrophosphatase in uncultivable hemotrophic mycoplasmas: identification and properties of the enzyme from *Mycoplasma suis. BMC Microbiol*, under review.

Zeder M, Van den Wyngaert S, Köster, O, **Felder KM** and Pernthaler J (2010): Automated quantification and sizing of unbranched filamentous cyanobacteria by model based object oriented image analysis. Appl Environ Microbiol. Epub ahead of print.

Hoelzle K, Doser S, Ritzmann M, Heinritzi K, Palzer A, Elicker S, Kramer M, **Felder KM** and Hoelzle LE (2009): Vaccination with the *Mycoplasma suis* recombinant adhesion protein MSG1 elicits a strong immune response but fails to induce protection in pigs. Vaccine 27(39):5376-82.

Felder KM, Hoelzle K, Wittenbrink MM, Zeder M, Ehricht R and Hoelzle LE (2009): A DNA microarray facilitates the diagnosis of *Bacillus anthracis* in environmental samples. Lett Appl Microbiol 49(3):324-31.

Hoelzle LE, Hoelzle K, Helbling M, Aupperle H, Schoon HA, Ritzmann M, Heinritzi K, **Felder KM** and Wittenbrink MM (2007): MSG1, a surface-localised protein of *Mycoplasma suis* is involved in the adhesion to erythrocytes. Microbes Infect 9(4):466-74.

Contribution to international conferences

2009, 7th Symposium of german speaking mycoplasmologists, Morschach, Switzerland. **Organisation** of Symposium.

2008, 12th International Symposium on Microbial Ecology (ISME), Cairns, Australia. **Poster** entitled “A DNA Microarray for the Identification of *Bacillus anthracis* in Environmental Samples”.

2008, 17th IOM congress in Tianjin, China. **Poster** entitled “Detection and Characterization of Carbohydrate-Metabolism Associated Virulence Factors of *Mycoplasma suis*”.

2008, biannual meeting of the division of Bacteriology and Mycology of the german veterinarian association, Braunschweig, Germany. **Poster** entitled “Charakterisierung der inorganischen Pyrophosphatase von *Mycoplasma suis*”.

2007, 6th Symposium of german speaking mycoplasmologists, Fürth, Germany. **Talk** entitled: “Detektion und Charakterisierung Kohlenstoffwechsel-assoziierter Virulenzfaktoren bei *Mycoplasma suis* – ein erster Standortbericht“.

2007, 66th annual congress of Swiss Society for Microbiology, Interlaken, Switzerland. **Talk** entitled “The glycolytic active *Mycoplasma suis* protein MSG1 is involved in the attachment to porcine erythrocytes”.

2006, 16th IOM congress in Cambridge, England. **Poster** entitled “Immunoblot and ELISA Based on Recombinant Proteins (MSG1, MSA1) for the Serological Diagnosis of *Mycoplasma suis* Infection”.

2006, biannual meeting of the division of Bacteriology and Mycology of the german veterinarian association, Wetzlar, Germany. **Talk** entitled “Nachweis von *Bacillus anthracis* in Umweltproben mittels DNA microarray“.

2006, 65th annual congress of Swiss Society for Microbiology, Lausanne, Switzerland. **Poster** entitled “A DNA Microarray for the Identification of *Bacillus anthracis* in Environmental Samples”.

11. References

1. S Razin: **Peculiar properties of mycoplasmas: the smallest self-replicating prokaryotes**. *FEMS Microbiol Lett* 1992, **79**:423-31.
2. NR Krieg: **Procaryotic Domains**. In: *Bergey's manual of Systematic Bacteriology* Edited by GM Garrity, vol. Two, Part A; 2005: 23.
3. W Ludwig, Schleifer, K.H., Whitman, W. B.: **Revised road map to the phylum Firmicutes**. In: *Bergey's Manual of Systematic Bacteriology* Edited by GM Garrity, vol. three: The Firmicutes; 2005: 1-2.
4. H Neimark, KE Johansson, Y Rikihisa, JG Tully: **Proposal to transfer some members of the genera *Haemobartonella* and *Eperythrozoon* to the genus *Mycoplasma* with descriptions of '*Candidatus* Mycoplasma *haemofelis*', '*Candidatus* Mycoplasma *haemomuris*', '*Candidatus* Mycoplasma *haemosuis*' and '*Candidatus* Mycoplasma *wenyonii*'**. *Int J Syst Evol Microbiol* 2001, **51**:891-9.
5. M Banai, I Kahane, J Feldner, S Razin: **Attachment of killed *Mycoplasma gallisepticum* cells and membranes to erythrocytes**. *Infect Immun* 1981, **34**:422-7.
6. SH Buttery, LC Lloyd, DA Titchen: **Acute respiratory, circulatory and pathological changes in the calf after intravenous injections of the galactan from *Mycoplasma mycoides* subsp. *mycoides***. *J Med Microbiol* 1976, **9**:379-91.
7. RA Daynes, JM Novak, BC Cole: **Comparison of the cellular requirements for human T cell transformation by a soluble mitogen derived from *Mycoplasma arthritidis* and concanavalin A**. *J Immunol* 1982, **129**:936-8.
8. ML Fernandez Guerrero, J Manuel Ramos, F Soriano: **Mycoplasma hominis bacteraemia not associated with genital infections**. *J Infect* 1999, **39**:91-4.
9. S Rosendal: **Pathogenetic mechanisms of Mycoplasma mycoides subsp. mycoides septicemia in goats**. *Isr J Med Sci* 1984, **20**:970-1.
10. ER Barreira, DC Souza, PF Goes, A Bousso: **Septic shock, necrotizing pneumonitis, and meningoencephalitis caused by Mycoplasma pneumoniae in a child: a case report**. *Clin Pediatr (Phila)* 2009, **48**:320-2.

11. BC Cole, GJ Sullivan, RA Daynes, IA Sayed, JR Ward: **Stimulation of mouse lymphocytes by a mitogen derived from *Mycoplasma arthritidis*. II. Cellular requirements for T cell transformation mediated by a soluble *Mycoplasma* mitogen**. *J Immunol* 1982, **128**:2013-8.
12. Y Naot, S Merchav, H Ginsburg: ***Mycoplasma neurolyticum*: a potent mitogen for rat B lymphocytes**. *Eur J Immunol* 1979, **9**:185-9.
13. MG Gabridge, Chandler, D.K.F., Daniels, M.J.: **Pathogenicity factors in mycoplasmas and spiroplasmas**. In: *The Mycoplasmas* Edited by S Razin, Barile, M.F., vol. 4; 1985: 313-352.
14. KB Waites, MF Balish, TP Atkinson: **New insights into the pathogenesis and detection of *Mycoplasma pneumoniae* infections**. *Future Microbiol* 2008, **3**:635-48.
15. V Schilling: ***Eperythrozoon coccoides*, eine neue durch Splenektomie aktionierbare Dauerinfektion der weissen Maus.** *Klin. Wochenschr.* 1928, **7**:1853-1855.
16. S Adler, Ellenbogen, V: **A note on two new blood parasites of cattle, *Eperythrozoon* and *Bartonella***. *J. Comp. Path. Ther.* 1934, **47**:219-221.
17. R Jensen: **Eperythrozoonosis in cattle and sheep of Louisiana. Preliminary report.** *Louisiana Bull* 1943, **388**:8.
18. EJ Splitter: **Eperythrozoon suis n. sp. and Eperythrozoon parvum n. sp., 2 new blood parasites of swine**. *Science* 1950, **111**:513-4.
19. LM Berent, JB Messick, SK Cooper, PK Cusick: **Specific in situ hybridization of *Haemobartonella felis* with a DNA probe and tyramide signal amplification**. *Vet Pathol* 2000, **37**:47-53.
20. JE Foley, S Harrus, A Poland, B Chomel, NC Pedersen: **Molecular, clinical, and pathologic comparison of two distinct strains of *Haemobartonella felis* in domestic cats**. *Am J Vet Res* 1998, **59**:1581-8.
21. RE Knutti, WB Hawkins: **I. Bartonella Incidence in Splenectomized Bile Fistula Dogs**. *J Exp Med* 1935, **61**:115-125.
22. EE Elko, W Cantrell: **Phagocytosis and anemia in rats infected with *Haemobartonella muris***. *J Infect Dis* 1968, **118**:324-32.
23. JP Kreier, M Ristic: **Genus III Haemobartonella; Genus IV Eperythrozoon**. In: *Bergey's Manual of Systematic Bacteriology*, vol. 1: Baltimore, MD: Williams & Wilkins; 1984: 724-729.

24. **Notification that new names and new combinations have appeared in volume 52, part 2, of the IJSEM. International Journal of Systematic and Evolutionary Microbiology**. *Int J Syst Evol Microbiol* 2002, **52**:691-2.
25. H Neimark, KE Johansson, Y Rikihisa, JG Tully: **Revision of haemotrophic *Mycoplasma* species names**. *Int J Syst Evol Microbiol* 2002, **52**:683.
26. JE Foley, NC Pedersen: **'*Candidatus* Mycoplasma *haemominutum*', a low-virulence epierythrocytic parasite of cats**. *Int J Syst Evol Microbiol* 2001, **51**:815-7.
27. B Willi, FS Boretti, V Cattori, S Tasker, ML Meli, C Reusch, H Lutz, R Hofmann-Lehmann: **Identification, molecular characterization, and experimental transmission of a new hemoplasma isolate from a cat with hemolytic anemia in Switzerland**. *J Clin Microbiol* 2005, **43**:2581-5.
28. JB Messick, PG Walker, W Raphael, L Berent, X Shi: **'*Candidatus mycoplasma haemodidelphidis*' sp. nov., '*Candidatus mycoplasma haemolamae*' sp. nov. and *Mycoplasma haemocanis* comb. nov., haemotrophic parasites from a naturally infected opossum (*Didelphis virginiana*), alpaca (*Lama pacos*) and dog (*Canis familiaris*): phylogenetic and secondary structural relatedness of their 16S rRNA genes to other mycoplasmas**. *Int J Syst Evol Microbiol* 2002, **52**:693-8.
29. JE Sykes, LM Ball, NL Bailiff, MM Fry: **'*Candidatus Mycoplasma haematoparvum*', a novel small haemotropic mycoplasma from a dog**. *Int J Syst Evol Microbiol* 2005, **55**:27-30.
30. H Neimark, W Peters, BL Robinson, LB Stewart: **Phylogenetic analysis and description of *Eperythrozoon coccoides*, proposal to transfer to the genus *Mycoplasma* as *Mycoplasma coccoides* comb. nov. and Request for an Opinion**. *Int J Syst Evol Microbiol* 2005, **55**:1385-91.
31. WC Stoffregen, DP Alt, MV Palmer, SC Olsen, WR Waters, JA Stasko: **Identification of a haemomycoplasma species in anemic reindeer (*Rangifer tarandus*)**. *J Wildl Dis* 2006, **42**:249-58.
32. H Neimark, KM Kocan: **The cell wall-less rickettsia Eperythrozoon wenyonii is a Mycoplasma**. *FEMS Microbiol Lett* 1997, **156**:287-91.
33. H Neimark, B Hoff, M Ganter: ***Mycoplasma ovis* comb. nov. (formerly *Eperythrozoon ovis*), an epierythrocytic agent of haemolytic anaemia in sheep and goats**. *Int J Syst Evol Microbiol* 2004, **54**:365-71.

34. H Neimark, A Barnaud, P Gounon, JC Michel, H Contamin: **The putative haemobartonella that influences *Plasmodium falciparum* parasitaemia in squirrel monkeys is a haemotrophic mycoplasma**. *Microbes Infect* 2002, **4**:693-8.

35. AP dos Santos, RP dos Santos, AW Biondo, JM Dora, LZ Goldani, ST de Oliveira, AM de Sa Guimaraes, J Timenetsky, HA de Morais, FH Gonzalez, et al: **Hemoplasma infection in HIV-positive patient, Brazil**. *Emerg Infect Dis* 2008, **14**:1922-4.

36. D Yang, X Tai, Y Qiu, S Yun: **Prevalence of *Eperythrozoon spp.* infection and congenital eperythrozoonosis in humans in Inner Mongolia, China**. *Epidemiol Infect* 2000, **125**:421-6.

37. Z Hu, J Yin, K Shen, W Kang, Q Chen: **Outbreaks of hemotrophic mycoplasma infections in China**. *Emerg Infect Dis* 2009, **15**:1139-40.

38. JF Zachary, EJ Basgall: **Erythrocyte membrane alterations associated with the attachment and replication of *Eperythrozoon suis*: a light and electron microscopic study**. *Vet Pathol* 1985, **22**:164-70.

39. K Groebel, K Hoelzle, MM Wittenbrink, U Ziegler, LE Hoelzle: ***Mycoplasma suis* invades porcine erythrocytes**. *Infect Immun* 2009, **77**:576-84.

40. JB Messick, G Smith, L Berent, S Cooper: **Genome size of *Eperythrozoon suis* and hybridization with 16S rRNA gene**. *Can J Microbiol* 2000, **46**:1082-6.

41. LE Hoelzle, K Hoelzle, M Ritzmann, K Heinritzi, MM Wittenbrink: ***Mycoplasma suis* antigens recognized during humoral immune response in experimentally infected pigs**. *Clin Vaccine Immunol* 2006, **13**:116-22.

42. LE Hoelzle, K Hoelzle, M Helbling, H Aupperle, HA Schoon, M Ritzmann, K Heinritzi, KM Felder, MM Wittenbrink: **MSG1, a surface-localised protein of *Mycoplasma suis* is involved in the adhesion to erythrocytes**. *Microbes Infect* 2007, **9**:466-74.

43. LE Hoelzle, K Hoelzle, A Harder, M Ritzmann, H Aupperle, HA Schoon, K Heinritzi, MM Wittenbrink: **First identification and functional characterization of an immunogenic protein in unculturable haemotrophic Mycoplasmas (*Mycoplasma suis* HspA1)**. *FEMS Immunol Med Microbiol* 2007, **49**:215-23.

44. K Hoelzle, J Grimm, M Ritzmann, K Heinritzi, P Torgerson, A Hamburger, MM Wittenbrink, LE Hoelzle: **Use of recombinant antigens to detect antibodies against *Mycoplasma suis*, with correlation of serological results to hematological findings**. *Clin Vaccine Immunol* 2007, **14**:1616-22.
45. K Hoelzle, S. Peter, M. Sidler, M.M. Kramer, M.M. Wittenbrink, K.M. Felder, L.E. Hoelzle: **Inorganic pyrophosphatase in uncultivable hemotrophic mycoplasmas: identification and properties of the enzyme from *Mycoplasma suis***. *BMC Microbiology* 2010.
46. **Anämie**. In: *Roche Lexikon*. München: Elsevier GmbH; 2003.
47. G Herold: **Anämie**. In: *Innere Medizin*; 1996: 22.
48. LE Bottiger, B Westerholm: **Acquired haemolytic anaemia. I. Incidence and aetiology**. *Acta Med Scand* 1973, **193**:223-6.
49. JW Semple, J Freedman: **Autoimmune pathogenesis and autoimmune hemolytic anemia**. *Semin Hematol* 2005, **42**:122-30.
50. G Herold: **Autoimmunhaemolytische Anämien (AIHA)**. In: *Innere Medizin* Edited by G Herold. Köln; 2006: 43-45.
51. CJ Elson, RN Barker: **Helper T cells in antibody-mediated, organ-specific autoimmunity**. *Curr Opin Immunol* 2000, **12**:664-9.
52. RN Barker, MA Vickers, FJ Ward: **Controlling autoimmunity--Lessons from the study of red blood cells as model antigens**. *Immunol Lett* 2007, **108**:20-6.
53. LD Petz: **Cold antibody autoimmune hemolytic anemias**. *Blood Rev* 2008, **22**:1-15.
54. JV Dacie: **Demonstration of antibodies to erytrhocytes 2.: Cold auto-antibodies**. In: *Immunological Methods* Edited by J Ackroyd. Oxford: Blackwell Scientific Publishing; 1964.
55. LE Hoelzle: **Haemotrophic mycoplasmas: recent advances in *Mycoplasma suis***. *Vet Microbiol* 2008, **130**:215-26.
56. J Wu, J Yu, C Song, S Sun, Z Wang: **Porcine eperythrozoonosis in China**. *Ann N Y Acad Sci* 2006, **1081**:280-5.
57. JB Messick: **Hemotrophic mycoplasmas (hemoplasmas): a review and new insights into pathogenic potential**. *Vet Clin Pathol* 2004, **33**:2-13.

58. M Ritzmann, J Grimm, K Heinritzi, K Hoelzle, LE Hoelzle: **Prevalence of *Mycoplasma suis* in slaughter pigs, with correlation of PCR results to hematological findings**. *Vet Microbiol* 2009, **133**:84-91.

59. JB Prullage, RE Williams, SM Gaafar: **On the transmissibility of *Eperythrozoon* suis by *Stomoxys calcitrans* and *Aedes aegypti***. *Vet Parasitol* 1993, **50**:125-35.

60. K Heinritzi, Plonait, H.: **Blutkrankheiten**. In: *Lehrbuch der Schweinekrankheiten* Edited by KH Waldmann, Wendt M.; 2004: 173.

61. A Jüngling: **Isolation, Charakterisierung und Bedeutung eines Kälteagglutinins bei der *Eperythrozoon suis*-Infektion des Schweines**. Munich: Ludwig-Maximilian University of Munich; 1989. PhD Thesis.

62. JF Zachary, AR Smith: **Experimental porcine eperythrozoonosis: T-lymphocyte suppression and misdirected immune responses**. *Am J Vet Res* 1985, **46**:821-30.

63. FE Boas, L Forman, E Beutler: **Phosphatidylserine exposure and red cell viability in red cell aging and in hemolytic anemia**. *Proc Natl Acad Sci U S A* 1998, **95**:3077-81.

64. D Bratosin, J Mazurier, JP Tissier, C Slomianny, J Estaquier, F Russo-Marie, JJ Huart, JM Freyssinet, D Aminoff, JC Ameisen, et al: **Molecular mechanisms of erythrophagocytosis. Characterization of the senescent erythrocytes that are phagocytized by macrophages**. *C R Acad Sci III* 1997, **320**:811-8.

65. D Bratosin, J Estaquier, F Petit, D Arnoult, B Quatannens, JP Tissier, C Slomianny, C Sartiaux, C Alonso, JJ Huart, et al: **Programmed cell death in mature erythrocytes: a model for investigating death effector pathways operating in the absence of mitochondria**. *Cell Death Differ* 2001, **8**:1143-56.

66. CP Berg, IH Engels, A Rothbart, K Lauber, A Renz, SF Schlosser, K Schulze-Osthoff, S Wesselborg: **Human mature red blood cells express caspase-3 and caspase-8, but are devoid of mitochondrial regulators of apoptosis**. *Cell Death Differ* 2001, **8**:1197-206.

67. KS Lang, PA Lang, C Bauer, C Duranton, T Wieder, SM Huber, F Lang: **Mechanisms of suicidal erythrocyte death**. *Cell Physiol Biochem* 2005, **15**:195-202.

68. F Lang, KS Lang, PA Lang, SM Huber, T Wieder: **Mechanisms and significance of eryptosis**. *Antioxid Redox Signal* 2006, **8**:1183-92.
69. M Foller, D Bobbala, S Koka, SM Huber, E Gulbins, F Lang: **Suicide for survival--death of infected erythrocytes as a host mechanism to survive malaria**. *Cell Physiol Biochem* 2009, **24**:133-40.
70. DS Kempe, A Akel, PA Lang, T Hermle, R Biswas, J Muresanu, B Friedrich, P Dreischer, C Wolz, U Schumacher, et al: **Suicidal erythrocyte death in sepsis**. *J Mol Med* 2007, **85**:273-81.
71. H Hamaguchi, H Cleve: **Solubilization of human erythrocyte membrane glycoproteins and separation of the MN glycoprotein from a glycoprotein with I, S, and A activity**. *Biochim Biophys Acta* 1972, **278**:271-80.
72. P Carranza, I Hartmann, A Lehner, R Stephan, P Gehrig, J Grossmann, S Barkow-Oesterreicher, B Roschitzki, L Eberl, K Riedel: **Proteomic profiling of Cronobacter turicensis 3032, a food-borne opportunistic pathogen**. *Proteomics* 2009, **9**:3564-79.
73. HU Lutz: **Homeostatic roles of naturally occurring antibodies: an overview**. *J Autoimmun* 2007, **29**:287-94.
74. G Biberfeld, E Gronowicz: ***Mycoplasma pneumoniae* is a polyclonal B-cell activator**. *Nature* 1976, **261**:238-9.
75. BiberfeldG, E Nilsson: **Mitogenicity of *Mycoplasma fermentans* for human lymphocytes**. *Infect Immun* 1978, **21**:48-54.
76. E Ruuth, F Praz: **Interactions between mycoplasmas and the immune system**. *Immunol Rev* 1989, **112**:133-60.
77. JW Simecka, SE Ross, GH Cassell, JK Davis: **Interactions of mycoplasmas with B cells: antibody production and nonspecific effects**. *Clin Infect Dis* 1993, **17 Suppl 1**:S176-82.
78. I Hoof, B Peters, J Sidney, LE Pedersen, A Sette, O Lund, S Buus, M Nielsen: **NetMHCpan, a method for MHC class I binding prediction beyond humans**. *Immunogenetics* 2009, **61**:1-13.
79. AP Kohm, KG Fuller, SD Miller: **Mimicking the way to autoimmunity: an evolving theory of sequence and structural homology**. *Trends Microbiol* 2003, **11**:101-5.
80. KW Wucherpfennig: **Mechanisms for the induction of autoimmunity by infectious agents**. *J Clin Invest* 2001, **108**:1097-104.

81. JE Smith, JE Cipriano, SM Hall: **In vitro and in vivo glucose consumption in swine eperythrozoonosis**. *Zentralbl Veterinarmed B* 1990, **37**:587-92.

82. K Kirk: **Membrane transport in the malaria-infected erythrocyte**. *Physiol Rev* 2001, **81**:495-537.

83. VB Brand, CD Sandu, C Duranton, V Tanneur, KS Lang, SM Huber, F Lang: **Dependence of *Plasmodium falciparum* in vitro growth on the cation permeability of the human host erythrocyte**. *Cell Physiol Biochem* 2003, **13**:347-56.

84. KS Lang, C Duranton, H Poehlmann, S Myssina, C Bauer, F Lang, T Wieder, SM Huber: **Cation channels trigger apoptotic death of erythrocytes**. *Cell Death Differ* 2003, **10**:249-56.

85. RK Wali, S Jaffe, D Kumar, N Sorgente, VK Kalra: **Increased adherence of oxidant-treated human and bovine erythrocytes to cultured endothelial cells**. *J Cell Physiol* 1987, **133**:25-36.

86. S Halbedel, C Hames, J Stulke: **Regulation of carbon metabolism in the mollicutes and its relation to virulence**. *J Mol Microbiol Biotechnol* 2007, **12**:147-54.

87. SR Schmidl, K Gronau, C Hames, J Busse, D Becher, M Hecker, J Stulke: **The stability of cytadherence proteins in *Mycoplasma pneumoniae* requires activity of the protein kinase PrkC**. *Infect Immun*, **78**:184-92.

88. D Bencina: **Haemagglutinins of pathogenic avian mycoplasmas**. *Avian Pathol* 2002, **31**:535-47.

89. JB Baseman, JG Tully: **Mycoplasmas: sophisticated, reemerging, and burdened by their notoriety**. *Emerg Infect Dis* 1997, **3**:21-32.